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GROWTH HORMONE GENE EXPRESSION AND TRANSLATION IN ECTOPIC TISSUES

by

CAROLINE LOUISE RENDER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY
EDMONTON, ALBERTA
SPRING, 1995

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To Fernando, Mum, Dad, Grandma, Grandad, Mae and Pai.

ABSTRACT

Until recently growth hormone (GH) was thought to be produced solely in somatotrophs of the pituitary gland. GH is, however, present in several extra-pituitary tissues of rats, although it is unclear if GH is synthesized in these ectopic sites, or bears a close structural homology to pituitary GH. The ability of neural and immune tissues of the domestic fowl to synthesize and secrete GH has therefore been investigated.

Immunoreactive GH (ir-GH) was detected in brain, hypothalamus, bursa of Fabricius, spleen and thymus by radioimmunoassay using specific antisera against chicken (c) GH. At least 4 size variants were observed when pituitary extracts were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, followed by immunoblotting with cGH antisera. In contrast, immunoblots of neural and immune tissue extracts revealed a single protein of 26 kDa, identical in size to the major GH fraction in the pituitary gland. This is larger than the predicted size of cGH, but is not due to post-translational glycosylation.

Northern blotting readily detected a single GH mRNA species in the pituitary gland, but not in neural or immune tissues. Transcription of the cGH gene in these ectopic tissues was, however, demonstrated using the Polymerase Chain Reaction (PCR), which is suited to detection of low abundance mRNA. The amplification of reverse transcribed mRNA in the presence of specific oligonucleotide primers that span a 689 base pair (bp) sequence of the cGH gene, generated single complementary deoxyribonucleic acid (cDNA) moieties of expected size. These cDNA fragments specifically hybridized with a cGH cDNA radiolabelled probe following Southern transfer. Digestion of these cDNAs with *Bam H1* produced two fragments of predicted size (465 bp and 224 bp), identical to those produced after endonuclease digestion of pituitary cDNA. Sequence analysis of the PCR products demonstrated 100% homology between exons 1 to 5 of chicken pituitary GH cDNA and the cDNA generated from neural and immune tissues.

These results therefore provide evidence for the expression and translation of a GH gene in neural and immune tissues of the domestic fowl and indicate that the same gene may be responsible for production of GH in all sites.

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LIST OF ABBREVIATIONS

5-HT:	serotonin
ACh:	acetylcholine
ACTH:	adrenocorticotropic hormone
AP-2:	activation protein 2
APUD:	amine precursor uptake and decarboxylation
BBB:	blood brain barrier
bGH:	bovine growth hormone
bp:	base pairs
cAMP:	cyclic adenosine monophosphate
cDNA:	complementary deoxyribonucleic acid
cGH:	chicken growth hormone
CNS:	central nervous system
con-A:	concanavalin A
CRE:	cAMP response element
CS:	chorionic somatomammotropin
CSF:	cerebrospinal fluid
DA:	dopamine
DNES:	diffuse neuroendocrine system
FSH:	follicle stimulating hormone
GH:	growth hormone
GHF-1/Pit-1:	pituitary specific transcription factor
GHF-2/Sp-1:	growth hormone factor 2
GHF-3:	growth hormone factor 3
GM-CSF:	granulocyte/macrophage colony stimulating factor
GRE:	glucocorticoid response element
GRF:	growth hormone releasing factor
hGH:	human growth hormone
hGH-N:	human GH normal gene
hGH-V:	human GH variant gene
ICC:	immunocytochemistry

icv:	intracerebroventricularly
IFN:	interferon
Ig:	immunoglobulin
IGF-I/II:	insulin-like growth factor I/II
IGHD:	isolated GH deficiency
IL-1/2:	interleukin-1/2
ip:	intraperitoneally
IQ:	intelligence quotient
-ir:	immunoreactive
ISH:	*in situ* hybridization
kb:	kilobase
kDa:	kilodalton
LH:	luteinizing hormone
MBH:	medio-basal hypothalamus
ME:	median eminence
mRNA:	messenger ribonucleic acid
NA:	nor-adrenaline
NF-1:	nuclear factor 1
NK:	natural killer cells
$O_2^{\cdot}$:	superoxide anion
oGH:	ovine growth hormone
PAGE:	polyacrylamide gel electrophoresis
pGH:	porcine growth hormone
PHA:	phytohaemagglutin
PRE:	proximal repressor element
PREB:	proximal repressor element binding protein
PL:	placental lactogen
PRL:	prolactin
REM:	rapid eye movements
rGH:	rat growth hormone
RIA:	radioimmunoassay
rtGH:	rainbow trout growth hormone

SCID:	severe combined immuno deficiency
sGH:	salmon growth hormone
SDS:	sodium dodecyl sulphate
SL:	somatolactin
SRIF:	somatostatin
SWS:	slow wave sleep
T_3:	L-3,5,3-triiodothyronine
tGH:	turkey growth hormone
TNF:	tumour necrosis factor
TRE:	T_3 response element
TRH:	thyrotropin releasing hormone
TSH:	thyroid stimulating hormone
TST:	total sleep time
USF:	upstream stimulatory factor

CHAPTER1. LITERATURE REVIEW

1.1. GENERAL OVERVIEW

The word pituitary is derived from the Greek *ptuo* ("to spit") and the Latin *ptuita* ("mucus") reflecting the opinion of early anatomists that the function of this organ was to siphon fluid from the brain and excrete it into the nose as mucus. However, it is now recognised that the pituitary gland secretes hormones which are essential to normal growth and metabolism, and the co-ordination of other endocrine glands. As early as 1912 a relationship between the pituitary gland and growth was being investigated. Hypophysectomy led to stunted growth of dogs and in addition caused an increased body fat content, decreased body temperature and reduced basal metabolic rate (BMR). The growth promoting principle of the pituitary gland indicated by Evans and Long in 1921 led to the discovery of the protein hormone, growth hormone (GH), which was purified from both bovine (Li and Evans, 1944), and human (Li and Papkoff, 1956), pituitary tissue. However, GH is no longer thought to be confined to the pituitary gland and growth promotion. Instead, it demonstrates a pleiotropy of actions, and appears to be produced in many extra-pituitary sites. This thesis will examine the ectopic production of GH in the brain and immune system of the chicken. Specifically, the expression and translation of the GH gene in these tissues will be studied.

1.2. PITUITARY GROWTH HORMONE

1.2.1. GH Secreting Cells

The distribution of GH secreting cells or somatotrophs within the adenohypophysis differs among different species (Elster,1993). A wide distribution of somatotrophs exists within the adenohypophysis of higher vertebrates, whereas they are often concentrated within specific regions of the gland in lower vertebrates. An example of this is seen in birds, where greater than 90% of the somatotrophs are confined to the caudal lobe, and the remainder located in the ventral region of the cephalic lobe (Mikami et al, 1986). Somatotroph cells are round, oval or triangular in shape, with well developed golgi and granular endoplasmic reticulae. They have a

large nucleus with heterochromatin, prominent nucleoli and numerous nuclear pores, and are packed with dense-core secretory granules (260-400nm in diameter) (Mason et al, 1993).

Growth hormone secretion from these cells is pulsatile in nature (Robinson, 1991) and under the control of the excitatory peptide GH-releasing factor (GRF) and the inhibitory peptide somatostatin (SRIF), both of which are released from the hypothalamus. Pulses of GH secretion from the anterior pituitary gland appear to be triggered by a rapid release of GRF into the portal system, preceded by, or coincident with a reduction in SRIF secretion. Growth hormone levels in the systemic circulation, are thus primarily regulated by the concentration of these inhibitory, and excitatory peptides in the portal vasculature. The control of GH secretion is, however, modified by a number of other important factors such as neurotransmitter levels, metabolic intermediates and other hormones and releasing factors.

1.2.2. GH Chemistry

The most abundant form of GH in the pituitary is a single chain 22 kilodalton (kDa) polypeptide with approximately 191 amino acids; the number varying slightly with species. The basic GH structure is highly conserved among mammalian species and contains four cysteine residues which form two disulphide bridges (Cys^{53}-Cys^{165} and Cys^{182}-Cys^{189}), linking large and small peptide loops respectively (Li and Dixon, 1971; Niall, 1971; Charrier and Martal, 1988).

Little, however, is known about the tertiary structure of GH, with the exception of X-ray crystal structural analysis of porcine GH (pGH) (Abdel-Meguid et al, 1987). Inspection of this structure revealed that 85 of the 191 amino acid residues (45%), compose a twisted bundle of four α-helices exhibiting distinct amphiphillic character (Cunningham et al, 1989; DeVos et al, 1991). Connections between the helices are formed by pieces of loop/coil structure of varying lengths, with a small portion of less well ordered structure at both the N and the C-terminus of the molecule (Beattie, 1993). In addition other small helical regions, non-colinear with the major helices, bring the total helical content of the pGH molecule to 52%.

Growth hormone has been isolated from chicken pituitary tissue (Farmer et al,

1974; Harvey and Scanes, 1977), with the monomeric form being most abundant. This protein has a molecular weight of 22-24 kDa based on electrophoretic mobility in sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), although estimates as high as 27 kDa have been reported (Lai et al, 1984; Souza et al, 1984). The sequence of a complementary deoxyribonucleic acid (cDNA) clone isolated from a chicken pituitary cDNA library, encodes a 25 amino acid leader peptide and a 191 amino acid mature protein with a calculated molecular weight of 22,225 daltons (Lai et al, 1984). The mature protein appears to share a considerable homology with rat (79%), bovine (76%) and human (58%) GH based on its messenger ribonucleic acid (mRNA) sequence (Souza et al, 1984; Scanes et al, 1986). In addition, the amino acid sequence obtained by sequential degredation of GH itself, indicates a similar homology (Lai et al, 1984; Leung et al, 1984). This includes the conservation of the four cysteine residues (Souza et al, 1984), important to structural folding. Other avian GH sequences have been investigated, including turkey and duck. The cDNAs coding for GH in these species, share 93% and 89% sequence homology respectively with the chicken (c) GH cDNA sequence (Foster et al, 1990; Goddard and Boswell, 1991). This similarity is especially pronounced in the nucleotide sequence for the leader polypeptide between chicken and turkey (97%). The amino acid sequence of turkey (t) GH has not been published, however, the mature peptide of duck GH is almost completely homologous to cGH, with only 3 conservative substitutions (Ser^{35} for Thr^{35}; His^{42} for Tyr^{42}; Lys^{139} for Arg^{139}), and a deletion of Ala^{50} (Goddard and Boswell, 1991).

1.2.3. GH Heterogeneity

Growth hormone in any one species is not a single protein but a family of multiple molecular variants (Lewis, 1984; Baumann, 1991). This heterogeneity stems from a variety of sources including multiple genes, post-transcriptional processing, and post-translational events. The heterogenous nature of human (Table 1.1.) and cGH (Table 1.2.) is detailed.

1.2.3.1. The GH Gene Family

The human (h) GH gene family consists of five highly homologous members (Seeburg, 1982), which are encoded within 55 kilobases (kb) on band q22-q24 of the long arm of chromosome 17 (George et al, 1981; Harper et al, 1982; Parks, 1989). These genes are characterized based on their resemblance to hGH or chorionic somatomammotropin (CS), also called placental lactogen (PL), and are orientated 5' to 3' hGH-N, hCS-L, hCS-A, hGH-V, hCS-B, separated by intergenic regions of 6 to 13 kb. Growth hormone and CS along with the more distantly related prolactin (PRL) are believed to have arisen from a common ancestral gene by gene duplication (Niall et al, 1971; Martial et al, 1979). Despite the high sequence identity, these genes are expressed selectively in two different tissues under different hormonal controls. The hGH-N gene encodes pituitary hGH and is expressed in the somatotroph cells of the anterior pituitary (Martial et al, 1979). This gene is similar in structure to other mammalian GH genes (Miller and Eberhardt, 1983), but is smaller than the cGH DNA (3.5 kb), which is located on chromosome 1 and contains expanded intron sequences (Tanaka et al, 1992). The hGH variant gene (hGH-V) has recently been shown to be expressed in the term placenta (Frankenne et al, 1987; Chen et al, 1989) and localized to the syncytiotrophoblast epithelium (Liebhaber et al, 1989). The other three genes in the family code for CSs, two of which are expressed along with hGH-V in the placenta (CS-A and CB-B), and a third, CS-L which is a pseudogene producing no mRNA (Walker et al, 1991). Any two of the five genes in the hGH family are at least 92% homologous (Parks, 1989), however, in subprimate mammals the CS genes appear to have arisen from duplication of the PRL gene and more closely resemble PRL (Wallis et al, 1992).

The recent isolation of a 26 kDa protein from the pituitary of the teleost *Gadus morhua* (atlantic cod) has identified a further member of the GH family (Rand-Weaver et al, 1991). This peptide, also found in other teleosts (rainbow trout, flounder, and salmon), has been named Somatolactin (SL) due to its structural resemblance to both GH and PRL (Ono et al, 1990; Rand-Weaver et al, 1992; Kaneko et al, 1993), and this protein shows a greater conservation than GH in teleosts (Takayama et al, 1991). The production of SL has been localized to cells in the pars intermedia of the

pituitary, which border the adjoining neurohypophyseal tissue (Kaneko et al, 1993). This is distinct from the localization of GH and PRL in the pars distalis. This protein does not, however, appear to be present in the pituitaries of higher vertebrates, such as the rat, frog or pigeon (Rand-Weaver et al, 1992). The function of SL is not yet established, but reproductive (Rand-Weaver et al, 1992), and hypercalcaemic (Kakizawa et al, 1993) actions have been proposed. Fish are also unusual in that salmon, rainbow trout and *Tilapia nilotica* pituitaries contain an additional GH gene, and the two GH genes are highly homologous (Agellon et al, 1988; Sekine et al, 1989; Male et al, 1992; Ber et al, 1993). The salmon genome also contains a GH pseudogene with a very similar nucleotide sequence to the GH gene, especially with respect to the intron sequences (Kavsan et al, 1994).

1.2.3.2. Transcriptional Heterogeneity

Two alternative promoters have been identified in the hGH gene; -54 (cap 2) and -197 (cap 3) base pairs (bp) from the transcription start site (cap 1) (Courtois et al, 1992), which would result in transcripts with novel exon 1 sequences. Putative TATA boxes have also been located upstream from these sites. Transcriptional heterogeneity may therefore result in the synthesis of heterogeneous proteins.

1.2.3.3. Post-transcriptional Heterogeneity

The second most abundant GH form in the human pituitary gland is a smaller 20 kDa protein, which accounts for approximately 10% of the total GH (Lewis et al, 1978). This protein arises by alternative splicing of the pre-mRNA, in which exon 2 is spliced to an alternative acceptor site located 45 bases within exon 3. This results in the synthesis of a 20 kDa hGH-N which lacks 15 internal amino acid residues (32-46 inclusive) (Lewis et al, 1980). The potential for an alternative splice site is conserved in the mouse (Linzer and Nathans, 1985), pig (Vize and Wells, 1987), rat (Barta et al, 1981) and bovine (Gordon et al, 1983) GH sequences. Thus, differential splicing of GH mRNA may not be confined to humans. While the disulphide bridges remain within the 20 kDa variant the tertiary structure of the protein remains to be elucidated.

Other additional alternate splicing products of the hGH-N gene have been

described in pituitary tumour tissue (Lecomte et al, 1987). One of these mRNA encodes a 17.5 kDa, 151 amino acid hGH, in which exon 2 is spliced directly to exon 4 and therefore lacks amino acid residues 32 to 71. The other mRNA predicts a 7.8 kDa protein. The proposed products of these mRNA transcripts have not been characterized, however, 16-18 kDa GH forms have been detected in murine and human pituitary extracts (Yoyoka and Friesen, 1986; Sinha and Jacobsen, 1988).

1.2.3.4. Post-translational Variants

Growth hormone heterogeneity is now well established (Baumann, 1991; Lewis, 1992) and reflects a family of peptides. This heterogeneity appears to be mainly derived from post-translational processing, and may represent a mechanism through which GH exerts its pleiotropic functions.

1.2.3.4.1. Deamidated GH

Growth hormone forms that are deamidated at Asp^{152} and Glu^{137} have been identified in human pituitary gland (Lewis et al, 1979; Lewis et al, 1981) and in the circulation (Baumann et al, 1983). These deamidated forms are also secreted by pituitary cells in culture (Baumann and MacCart, 1982). Deamidation has also been shown to contribute to GH heterogeneity in cattle (Baumann et al, 1983; Secchi et al, 1986).

1.2.3.4.2. Phosphorylated GH

A phosphorylated form of GH has been described in rat (Liberti and Joshi, 1986), sheep (Liberti et al, 1985) and chicken (Aramburo et al, 1989a), indicating a further source of GH heterogeneity. Both rat and chicken pituitary cells in culture are capable of incorporating ^{32}P-phosphate into a GH-immunoreactive (-ir) protein (Liberti and Joshi, 1986; Aramburo et al, 1990a). In addition, native and recombinant cGH and tGH can be phosphorylated *in vitro* by the catalytic subunit of protein kinase A and [γ^{32}-P]ATP (Aramburo et al, 1990a; Aramburo et al, 1992). Human GH is susceptible to phosphorylation by a tyrosine kinase (Baldwin et al, 1983) and purified atlantic salmon (s) GH, by a calmodulin dependent protein kinase (Skibeli et al,

1990). Phosphorylation is a modification that would make a protein more acidic, and charge heterogeneity has been demonstrated in sGH. This appears to be related to its phosphate content since treatment with alkaline phosphatase eliminates most of the charge variants (Skibeli et al, 1990). A similar situation may exist with avian GH, as cGH has up to ten charge variants (Houston and Goddard, 1988; Aramburo et al, 1990b; Montiel et al, 1992), at least one of which is lipolytic (Aramburo et al, 1989b).

1.2.3.4.3. Glycosylated GH

The existence of glycosylated forms of GH has been demonstrated in a number of species, including the pig (Sinha et al, 1990), mouse (Sinha and Jacobsen, 1987), rat (Bollengier et al, 1989), and chicken (Berghman et al, 1987; Aramburo et al, 1991). However, the structure of glycosylated GH and its constituent oligosaccharides has not been determined. A glycosylated hGH may also exist in the pituitary gland (Sinha and Lewis, 1986), but the hGH-N gene and its variants do not contain a concensus sequence for N-glycosylation (Baumann et al, 1991). However, this does not rule out the possibility of O-linked carbohydrates. N-glycosylated GH-like proteins do, however, appear to be present in the pituitary but may represent expression of the hGH-V or another unidentified gene (Diaz et al, 1993), since the hGH-V can be glycosylated at the N-terminus (Ray et al, 1989).

1.2.3.4.4. Acetylated GH

A portion of the 22 kDa hGH has an acyl group (probably acetyl) at its N-amino terminus (Lewis et al, 1979). This addition does not appear to affect the bioactivity or immunoreactivity of the protein when compared to 22 kDa hGH (Lewis et al, 1979). However, acetylation does affect the isoelectric point and consequently the electrophoretic mobility of this variant. As such, it has also been named "fast GH".

1.2.3.4.5. Oligomers

Size variants of GH found in the pituitary gland include dimers and higher

oligomers (up to pentameric GH) (Stolar et al, 1984). Indeed certain GH forms, such as the 20 kDa hGH, bovine (b), ovine (o) and pGH, have a natural tendancy to dimerize (Baumann, 1991). Most investigations have centered around the dimeric or "big" GH which is thought to consist of two 22 kDa monomers. These may be covalently linked by a disulphide bridge between Cys^{35} in one molecule and Cys^{165} in the other, as well as a second linkage between Cys^{182} and Cys^{189} (Lewis et al, 1977). Approximately 2% of GH oligomers are covalently linked by bonds other than disulphide bridges (Benveniste et al, 1975; Lewis et al, 1977; Stolar et al,1984). Alternatively, other non-covalently linked dimers have been described, including 20 kDa homodimers, 22 kDa homodimers and 20/22 kDa heterodimers (Chapman et al, 1981; Brostedt and Roos, 1989).

Oligomeric GH is generally believed to have diminished bioactivity and immunoreactivity. The growth promoting activity of dimeric GH is only 10-20% of monomeric 22 kDa GH (Li et al, 1974; Becker et al, 1987), yet lipolytic and lactogenic activities range from 30-250%, depending on the type of dimer (Li et al, 1974; Lewis et al, 1977; Brostedt et al, 1990). Dimeric GH binds to the GH receptor with only 25-65% affinity of monomeric species (Benveniste et al, 1975; Soman et al, 1977), which may explain its reduced activity. However, Li et al (1974) reported full immunoreactivity of dimeric GH, and this was confirmed by Brostedt et al (1990), thus indicating that the constituent components are fully immunoreactive when associated.

1.2.3.4.6. GH Fragments

A 5 kDa GH fragment has been described in pituitary tissue, formed by cleavage between amino acid residues 43 and 44 (GH_{1-43}) (Singh et al, 1983; Nicoll et al, 1986). This fragment does not appear to be growth promoting (Nicoll et al, 1986; Stewart et al, 1992), but does possess insulin-potentiating effects in hypophysectomized mice and rats (Frigeri et al, 1988; Salem, 1988). The remaining fragment GH_{44-191} has not been definatively identified, but GH-ir species corresponding to its predicted size of approximately 17 kDa have been identified in pituitary extracts (Sinha and Jacobsen, 1988; Lewis et al, 1991).

Cleaved GH fragments with reduced electrophoretic mobility have also been described in pituitary extracts (Chambrach et al, 1973; Singh et al, 1974). These GH variants migrate at 24 kDa in SDS gels due to molecular unfolding of the large loop of the GH molecule. The cleavage points were identified as Arg^{134}, Lys^{140} and Lys^{145} and unfolding results in two chain GH forms connected by disulphide bridges (Singh et al, 1974). These variants are probably non-native, but products of degradation pathways or extraction procedures (Baumann, 1991).

1.2.4. GH Biosynthesis

Growth hormone synthesis in human somatotroph cells is under the control of general and tissue specific transcription factors, hormones and second messengers and has recently been reviewed by Theill and Karin (1993).

1.2.4.1. Promoter Structure and Regulation

The coding region of the hGH gene is preceeded by 500 bp of DNA sequences called the promoter, which confers on the gene its cell specific expression and sensitivity to hormonal control. The promoter region contains DNA sequences or *cis*-acting elements which bind regulatory *trans*-acting factors that exert a combination of positive and negative influences on the GH gene (Karin et al, 1990a; Pan et al, 1990). Certain *trans*-acting factors which bind the GH promoter are ubiquitous whilst others seem pituitary specific (Bodner and Karin, 1987; LeFevre et al, 1987).

Upon binding to the promoter these transcription factors facilitate the entry of RNA polymerase II, the enzyme which transcribes the GH gene. This produces a pre-mRNA of approximately 1300 nucleotides containing both intron and exon sequences. The pre-mRNA is capped at its 5' end by 7 methyl-guanine triphosphate and methylation, and polyadenylated at the 3' end (Parks et al, 1989). Production of the mature mRNA is achieved by removing the intron sequences. This splicing generates a pre-hGH molecule which is proteolytically cleaved to 22 kDa hGH.

1.2.4.2. GH Gene Regulation by Ubiquitous *Trans*-acting Factors

The technique of DNA footprinting has identified a protected region in the

hGH promoter analogous to sequences in other human genes which bind upstream stimulatory factor (USF). This footprint, located between -257 /-290, also binds another factor, as USF occupies only the proximal region between nucleotides -257/-267 (Lemaigre et al, 1989a). Nuclear factor-1 (NF-1) has been suggested as the corresponding *trans*-activator (Lemaigre et al, 1989a) binding to the distal region of this *cis*-acting element (-268/-290). Collectively referred to as GH factor-3 (GHF-3), in rats this factor binds between nucleotides -219/-239 and consists of at least 5 different complexes, each with a common DNA binding subunit (Schaufele et al, 1990a). This common DNA binding subunit may act as a docking site for other transcriptional proteins, which would greatly increase the plasticity of the rat (r) GH promoter if each of the complexes were to have different transcriptional effects.

The proximal region of this GHF-3 footprint (-263/-290) can also be bound by another ubiquitous factor, activation protein-2 (AP-2) (Courtois et al, 1990), indicating competition between NF-1 and AP-2 for overlapping sites in the GH promoter. A further *cis*-element binding the ubiquitous factor Sp-1 (or GHF-2) was discovered by Lemaigre et al (1989b). This transcription factor binds between nucleotides -116/-140, which corresponds to the GC box described in several viral and eukaryotic gene promoters (Lemaigre et al, 1989b).

These ubiquitous *trans*-acting factors are responsible for the basal activity of the GH gene and can stimulate transcription from the promoter *in vitro* when derived from non-pituitary cell extracts, indicating that these factors are not restricted to the pituitary gland.

1.2.4.3. Pituitary Specific Expression

A unique protein-binding region has been identified in the first 260 bp of the rat (Nelson et al, 1986; West et al, 1987; Ye et al, 1987) and human (Cattini et al, 1986b) GH promoter regions of GH expressing cells exclusively, even though this promoter shares a 60% homology with the rest of the GH family (Parks, 1989). This finding indicates the presence of a factor important to cell specific expression of the GH gene.

This pituitary specific transcription factor, cloned by 2 groups and named

GHF-1 (Bodner et al, 1988) or Pit-1 (Ingraham et al, 1988), has been shown to stimulate GH transcription *in vitro* in both GC cells (a pituitary tumour cell line) and cells that do not normally express GH (HeLa cells) (Bodner and Karin, 1987; Fox et al, 1990). Specific inhibition of Pit-1/GHF-1 synthesis by complementary oligonucleotides leads to a marked decrease in GH expression (Castrillo et al, 1991), and naturally occuring mutations that cause pituitary dwarfism in mice, have been shown to reside in Pit-1/GHF-1 on chromosome 16 (Li et al, 1990). The appearance of Pit-1/GHF-1 protein in the pituitary gland of mice shows good temporal and spatial correlation with activation of the GH gene. This timing suggests that accumulation of Pit-1/GHF-1 transcripts could mark the commitment step in the somatotropic differentiation pathway during development of the pituitary gland (Dolle et al, 1990). Thus, activation of Pit-1/GHF-1 translation is probably responsible for conferring somatotropic cell identity (Simmons et al, 1990) and indicates why dwarf mice have no specific pituitary cell types. All this evidence points to Pit-1/GHF-1 being an important regulator of GH.

Pit-1/GHF-1 binds to 2 sites upstream of the hGH TATA-box, located around positions -105/-132 and -66/-94. Both of these sites were found to be important promoter elements *in vivo* (Lefevre et al, 1987) and *in vitro* (Bodner and Karin, 1987). Pit-1/GHF-1 is a 33 kDa polypeptide (Castrillo et al, 1989) which recognizes the nucleotide binding motif TTATG/CCAT. This peptide is thought to be a member of the homeodomain proteins (Bodner et al, 1988) as shown by the 73% cDNA sequence homology of its carboxy terminus to a concensus homeodomain (Karin et al, 1990a). This factor deserves classification into a further subgroup - the POU-specific domain proteins for its homology in a different region (>90%) to Oct-1 and Unc-86, the transcriptional activators involved in development (Rousseau, 1992).

Both the POU and the homeodomain regions of the transcription factor appear to be involved in DNA binding (Bodner et al, 1988). A recent finding also indicated that the POU-specific domain was involved in bending of DNA, distorting it from its regular double helical structure (Verrijzer et al, 1991). This event may be sufficient for activation of transcription. Alternatively, it may be part of the mechanism by which the POU-specific domain participates in protein-protein interactions (Karin et al,

1990b), stabilizing the Pit-1/GHF-1 DNA complex or bringing together a highly organized functional nucleoprotein structure at the origin of DNA replication (Verrijzer et al, 1991).

Pit-1/GHF-1 alone, however, is not efficient in pituitary GH gene activation and only directs minimal expression in transgenic animals (Lira et al, 1993). Additional factors, acting at distinct promoter elements beyond the Pit-1/GHF-1 binding sites, may act in a synergistic fashion with Pit-1/GHF-1 to regulate endogenous gene activation in the pituitary gland. An example of this type of synergism is demonstrated by the actions of Sp-1 and Pit-1/GHF-1. These two transcription factors have overlapping binding sites within the GH promoter (Schaufele et al, 1990b). However, structural integrity of both binding sites appears essential to maximal expression of the rGH promoter after transfection into pituitary GC cells. In this case it appears that a multistage mechanism is involved in promoter activation.

1.2.4.4. Tissue Specific Repression

In addition to the positive effects of Pit-1/GHF-1, negative elements contribute to the cell type specific expression of the rGH gene. Two silencer elements located approximately 300bp and 500bp upstream of the mRNA start site, function to decrease promoter activity in non-pituitary cells such as the CV-1 line, but have little or no effect on expression in pituitary cells (Larsen et al, 1986a; Larsen et al, 1986b; Konzak and Moore, 1992). *Trans*-acting factors binding to these regions have been isolated (Factor I and Factor II) but do not correspond to any known proteins (Peritz et al, 1988).

The ubiquitous transcription factors NF-1 and AP-2 may be in part responsible for repression of GH gene expression. These factors bind to a silencer element located around -266/-309 in the promoter sequences (Guerin et al, 1993). Deletion of this distal region of the GH promoter and also a proximal repressor element (PRE) located -152/-169, causes an increased expression of enzyme genes in a CAT-transfection assay (Pan et al, 1990). A proximal repressor element binding protein (PREB) which binds to the PRE has been identified (Pan et al, 1990), and this factor is present only in cell lines which were not derived from the pituitary.

These repressor or silencer elements, which are found only in non-pituitary cell lines (Roy et al, 1992) therefore appear to prevent expression of the GH gene in inappropriate cell types, and the factors binding them may be cell-specific or ubiquitous in nature. A further mechanism for extinction of GH expression exists, since fusion of non-GH expressing cells with pituitary derived GH_3 cells results in the loss of GH production (Strobl et al, 1982; McCormick et al, 1988). This loss could not be attributed to any direct repressor effect but was accompanied by loss of Pit-1/GHF-1 protein and mRNA expression (McCormick et al, 1988). Thus, the lack of GH production in tissues other than pituitary may be due to the absence of the transactivator Pit-1/GHF-1.

Methylation of DNA at specific cytosine bases is another mechanism affecting gene transcriptional activity, and a tissue specific correlation between hypomethylation and gene activity has been reported for hGH (Hjelle et al, 1982). Rat GH gene expression was correlated with an unmethylated Tha 1 site (CGCG), located 144bp upstream of the GH mRNA transcription initiation site. This site was entirely methylated in DNA prepared from non-GH producing tissues (spleen, kidney, liver, brain) (Moore et al, 1982). In addition to this tissue specific effect, blocking the action of methyl-transferase can increase GH mRNA levels 3-8 fold in GH_3 cells (Lan et al, 1984), and demethylation by 5-azacytidine is correlated with increased gene expression (Laverriere et al, 1988). This effect is likely on the 5' flanking DNA of the GH promoter (Gaido et al, 1989) as has been demonstrated for many other genes (Busslinger et al, 1983).

1.2.4.5. Hormonal Regulation of GH Gene Expression

1.2.4.5.1. GRF

Growth hormone releasing factor specifically increases the synthesis of pituitary GH as well as steady state levels of GH mRNA in both normal rat pituitary cell cultures (Gick et al, 1984; Chomczynski et al, 1988), and human somatotroph tumour cells (Davis et al, 1989; Herman et al, 1990). Growth hormone releasing factor can also stimulate GH mRNA content of rat somatotroph cells *in vivo* (Morel et al, 1989; Hu et al, 1993) but has no effect on GH mRNA in rat pituitary tumour cells (Zeytin et

al, 1984). This effect could be due to an increase in GH gene transcription or increased stability of the GH mRNA, and GRF has been shown to activate transcription of the rGH gene both *in vivo* and *in vitro* (Barinaga et al, 1983; Barinaga et al, 1985).

Regulation of hGH gene expression by GRF involves cyclic adenosine monophosphate (cAMP) as a second messenger (Barinaga et al, 1983; Bilezikjian and Vale, 1983; Clayton et al, 1986; Dana and Karin, 1989). The *cis*-element involved in the cAMP stimulation of hGH transcription appears to reside between nucleotides -9/-82 (Dana & Karin, 1989). This cAMP response element (CRE) does not possess the concensus sequence described in other genes, instead a novel CRE in the proximal promoter region of hGH probabaly participates in cAMP mediated transcription. In addition, the 5' flanking DNA of the rGH gene can mediate stimulation by cAMP (10 to 20 fold). The cAMP responsive region was mapped to sequences between -104/+11 which also contain the proximal cell specific element important for cell specific expression (Copp et al, 1989). Again this region does not contain the concensus cAMP regulatory element (TGACGTCA), but the effect of cAMP appears to be synergistic with that of L-3,5,3'-tri-iodothyronine (T_3), suggesting a co-operative effect of the distinct thyroid hormone responsive and CRE regions to activate rGH gene expression.

1.2.4.5.2. SRIF

Somatostatin, while having no effect on GH mRNA production in human pituitary tumour cells (Levy and Lightman, 1988; Herman et al, 1990), bovine pituitary cells (Tanner et al, 1990), trout pituitary cells (Yada and Hirano, 1992) or rat pituitary gland (Morel et al, 1989), may antagonize the effects of GRF on GH mRNA synthesis. Somatostatin has previously been shown to inhibit adenyl cyclase activity and attenuate cAMP accumulation in pituitary cells (Bilezikjian and Vale, 1983). By this mechanism, SRIF may be able to reduce GH transcription. An additional study by Sugihara et al (1993), indicates that SRIF may reduce transcription of the GH gene in rats by reducing steady state levels of GRF.

1.2.4.5.3. Thyroid Hormones

Both *in vivo* and *in vitro* observations indicate the critical importance of T_3 in the stimulation of rGH gene expression. Hypothyroidism induced by injection of ^{131}I in rats was found to be associated with a fall in GH mRNA levels which was reversed by thyroid hormone replacement (Franklyn et al, 1986). In this species, thyroidectomy leads to a dramatic reduction of GH production and GH mRNA levels, and thyroid hormone therapy restores both these parameters to normal (Mirrell et al, 1987; Wood et al, 1987b). *In vitro*, T_3 has been shown to stimulate GH gene expression in normal rat pituitary gland (Chomczynski et al, 1988), and increase accumulation of cytoplasmic GH mRNA in cultured rat pituitary tumour cells (Wegnez et al, 1982). The stimulatory effect of T_3 on rGH mRNA production is associated with an increase in transcriptional activity of the GH gene which is proportional to the concentration of thyroid hormone-receptor complexes (Yaffe and Samuels, 1984). The T_3-T_3 receptor complex is thought to stimulate GH transcription by interacting with T_3 responsive elements (TRE) located in the 5' region, between nucleotides -254/-163 and in the third intron of the rGH gene (Casanova et al, 1985; Larsen et al, 1986b; Norman et al, 1989). The binding site in the 5' region includes 2 copies of a 7bp direct repeat, the centres of which are separated by 10bp (Koenig et al, 1987). The DNA sequence specific binding of the receptor did not require occupancy of the receptor by the hormone T_3 (Lavin et al, 1988). Thus, T_3 may act by stimulating a transcriptional activational function rather than by stimulating DNA binding. Human T_3 receptor has been shown to specifically interact with a segment located -129/-290 in the hGH promoter (Barlow et al, 1986). Within these two promoters, conserved regions exist between nucleotides -173/-188 in hGH and -191/-206 in rGH (Morin et al, 1990). However, the role of thyroid hormones in hGH gene expression is less clear. Although most studies indicate a stimulatory effect of thyroid hormone on hGH secretion (Cattini et al, 1986b; Chomczynski et al, 1993), Cattini and Eberhardt (1987) reported that T_3 had no effect on the hGH promoter in an expression vector containing 500bp of hGH 5' flanking sequences. Even within 2.7kb of 5' flanking sequences, the hGH promoter appeared unresponsive to T_3 (Brent et al, 1988).

1.2.4.5.4. Glucocorticoids

Glucocorticoids can stimulate production of GH mRNA in rat pituitary cell lines (Bancroft et al, 1969; Martial et al, 1977; Spindler et al, 1982; Nyborg et al, 1985). This increase may arise in part from a post-transcriptional event such as glucocorticoid induced stabilization of GH mRNA (Diamond and Goodman, 1985), but evidence suggests that a significant induction of GH gene transcription is involved (Evans et al, 1982; Karin et al, 1984).

Glucocorticoid stimulation of gene transcription is mediated by the binding of glucocorticoid hormone-receptor complexes to multiple short DNA sequences, glucocorticoid response elements (GRE). These are usually contained within the first 500 nucleotides of the 5' flanking sequences of mammalian genes (Robins et al, 1982). Inspection of a series of GREs in over twenty known glucocorticoid inducible promoters indicated a possible GRE concensus sequence in the hGH gene promoter (Beato, 1989). An additional functional GRE in the first intron of the gene has also been identified, approximately 100bp downstream from the start of hGH gene transcription (Moore et al, 1985). This site is not conserved in the rGH gene (Slater et al, 1985), however, sequences lying 3' to the transcription start site can mediate glucocorticoid induction of rGH gene transcription (Birnbaum and Baxter, 1986).

1.2.4.6. Evolutionary Conservation of Transcriptional Regulation

The transcriptional regulation of the GH gene appears to be conserved in vertebrate evolution. The GH genes of both *Tilapia nilotica* (Ber et al, 1992) and rainbow trout (rt) (Argenton et al, 1993) contain sequences which resemble those of the binding domain for the transcription factor Pit-1/GHF-1. In addition, nuclear proteins from rat GC cells and from trout pituitaries were found to specifically interact with one of these regions. This suggests that rat Pit-1/GHF-1 binds to and activates the rtGH promoter (Argenton et al, 1993). Recently, a fish Pit-1/GHF-1 cDNA has been cloned and found to bind specifically to at least four sites in the rtGH promoter and activate gene expression (Yamada et al, 1993). The promoter region of the salmon SL gene, which belongs to the GH family, also contains at least five Pit-1/GHF-1 binding sites. The concensus sequence of these binding sites closely matches

the 9 bp motif (T/A)(T/A)TATNCAT recognised by rat Pit-1/GHF-1 (Yamada et al, 1993). The promoter region is highly conserved among fish genes, and thus the basic mechanisms regulating pituitary GH gene transcription appear to have been conserved between fish and mammals.

The promoter region of the cGH gene, however, has no overall homology with the corresponding regions of mammalian genes, and does not appear to contain any concensus sequences for hormonal regulation. However, it does contain a short sequence between nucleotides -91 and -113, which is highly homologous to the antisense strand sequence of the proximal binding site for the pituitary specific transcription factor Pit-1/GHF-1 in the rGH gene (Tanaka et al, 1992). Sixteen out of 23bp in this region are identical between chicken and rat genes, strongly suggesting the pituitary specific expression of avian GH genes is also regulated by factors like Pit-1/GHF-1.

1.3. ECTOPIC HORMONE SYNDROMES

Each hormonal peptide was traditionally considered to be a unique product of a single cell type, localized to a limited region of an endocrine gland (Roth et al, 1982). It is, however, now known that a single cell may produce many hormones and that these cells may not be confined to a single tissue or organ. This is perhaps not surprising, since every cell has the same genetic complement and thus the potential to produce every protein encoded by its DNA sequences. Under physiological conditions, cell-specific protein production results from DNA repression, although this is often attenuated or 'derepressed' in pathophysiological states characterized by ectopic hormone production (Melmed and Rushakoff, 1987) (Table 1.3.). One of the prominent features of carcinomas, for example, is the synthesis of specialized proteins not usually expressed by the cell, and this ectopic secretion of hormones is associated with a variety of tumours. Although original reports of these syndromes described primarily lung carcinomas, carcinoids, thymomas and fibrosarcomas, virtually all tumours have the potential of hormone secretion. Nevertheless, the frequency of occurence of ectopic hormone secretion among various tumour types is not random. Growth hormone for instance, is known to be produced in ovarian

teratoma tissue (Pilavdzic et al, 1993), metastatic pancreatic tumours (Ezzat et al, 1993), and pharangeal pituitary tissue (Corenblum et al, 1980; Warner et al, 1982), but appears to be more common in lung carcinoma (Beck and Burger, 1972; Greenberg et al, 1972). In some instances gigantism or acromegaly may be present due to the excessive GH production (Lindholm et al, 1975; Melmed and Rushakoff, 1987), but occasionally, production of GRF secondary to GH by the tumour results in stimulation of GH secretion by the pituitary (Melmed et al, 1985).

Although little is known about the mechanisms involved in GH production by ectopic tumours, pituitary adenomas secreting GH are usually monoclonal in origin. They are derived from a single mutated mother cell, indicating that the cause of tumour formation resides at the DNA level and is not due to dysregulation by external factors (Adams et al, 1993). The mutation in these tumours may occur in the coding region for the alpha-s (Landis et al, 1989) subunit of the Gs protein controlling adenyl cyclase activity. This is seen in 30-40% of GH secreting tumours, and results in elimination of the intrinsic GTPase activity, thus ultimately leading to constituitive adenyl cyclase activity and cAMP production. The resultant cAMP production is presumed to lead to GH hypersecretion and excessive somatotroph cell proliferation. The GH gene in GH secreting pituitary tumours is also less methylated than the GH gene in normal tissue (Adams et al, 1993). Since reduced methylation has been shown to increase GH gene expression (Lan, 1984), this may explain the GH over-expression in some GH secreting pituitary tumours. These mechanisms may also play a role in the secretion of GH from ectopic tumours. Greenberg et al (1972), found that dibutyryl cAMP increased release of ir-GH from lung carcinoma cells, suggesting that cyclic nucleotides may be involved in GH release from this ectopic site.

1.3.1. The Diffuse Neuroendocrine System

The realization that '*everything is produced everywhere and does everything*' led to the conceptual development of the diffuse neuroendocrine system (DNES) (Pearse, 1984). This system encompasses the neural, endocrine, neuroendocrine and immune systems, which separately utilize the same chemical messengers for neurocrine, neuroendocrine, endocrine, cytokine, paracrine, autocrine and intracrine

signalling in homeostatic regulation. It has therefore been suggested that these tissues may have a common embryological origin.

In terms of phylogenesis, a pluripotential ancestral neuron, derived from an undifferentiated and pluripotential cell was probably a versatile structure, equally endowed for the dispatch of long-distance and short-distance chemical messages (Pearse, 1978). This neuron was thus obliged to carry out all the endocrine functions of the organism and in this respect closely resembles the classical neuroendocrine cells of the hypothalamus, as well as cells of the APUD (or 'amine precursor, uptake and decarboxylation') series. The APUD concept arose after extensive work on the cytochemical characterization of known and probable endocrine cells. These cells were found to possess the properties of both 'amine handling' and peptide hormone production, whatever their localization in the body (Pearse, 1984). APUD cells are widely distributed, and include cells of the hypothalamo-pituitary axis, pineal gland and gastro-intestinal tract. The somatotroph cells of the anterior pituitary gland are members of this series and along with the hypothalamus share a common origin from specialized ectoderm of the neural ridge or neuroectoderm (Takor-Takor and Pearse, 1975). However, many of the APUD cells are not derived from the neuroectoderm. These include pancreatic B cells and intestinal cells producing substance P and motilin, which are derived from the endoderm (Pearse, 1979). Their inclusion in the APUD category is due to their amine handling capability, which appears to be present at some stage of the development of all cells of the DNES, and is due to possession of the enzyme DOPA decarboxylase (Pearse, 1984). Thus the wide distribution of these APUD cells, the proposed common origins of some but not all of these cells, and their capacity to produce peptide hormones, may explain the presence of peptides such as GH outside their specialized glandular locale.

1.4. NEURAL GROWTH HORMONE

There is growing evidence that most of the hormones originally isolated from the anterior pituitary gland are also present within the central nervous system (CNS) (Krieger, 1980; Krieger, 1983). Consistent with this trend, GH has been identified in the brain of several species by radioimmunoassay (RIA), immunocytochemistry (ICC)

and *in situ* hybridization (ISH).

1.4.1. Distribution

The first suggestion that GH was located in the CNS was made by Linfoot et al (1970), who identified GH in the cerebrospinal fluid (CSF) by RIA. Since then GH-ir has been demonstrated in whole brain homogenates and appears to be readily extractable from all brain tissues (Pacold et al, 1978; Kyle et al, 1981; Hojvat et al, 1982a). Further ICC studies have revealed a widespread distribution of GH-ir in neuronal fibres of hypothalamic nuclei, hippocampus, amygdala, thalamus, mesencephalon, pons, medulla, spinal cord and posterior pituitary gland of the rat (Lechan et al, 1981). In addition, neuronal perikarya and axon terminals in the hypothalamus were found to be positive for GH-ir (Lechan et al, 1981), with thyrotropin-releasing hormone (TRH) immunoreactivity having an identical distribution (Lechan et al, 1983). Growth hormone immunoreactivity has also been identified in the brain of freshwater fish, in neurons of the pre-optic nucleus which give rise to seperate beaded axons terminating in the infundibulum, posterior pituitary and third ventricle (Hansen and Hansen, 1982). A similar distribution is also seen in the immature sea lamprey (Wright , 1986), even though no GH-ir could be demonstrated in the adenohypophysis, thereby indicating a purely central function for GH in this species.

1.4.2. Origins

The information gained from immunological methods, however, does not shed sufficient light on the origins of this GH-ir substance. Anterior pituitary hormones were until recently viewed solely as peripheral hormones and essentially excluded from the CNS by the blood brain barrier (BBB). Several reports supporting the impermeability of the BBB to GH exist. For example, radiolabelled GH uptake into fetal and adult brain could not be demonstrated (Pacold et al, 1978; Hojvat et al, 1982a). In addition, situations leading to increased plasma GH concentration were not mirrored by similar changes in the CSF of rhesus monkeys (Belchetz et al, 1982). In contrast, some authors have shown uptake from the systemic circulation (Stern et al,

1975a). As well, aluminium was shown to specifically enhance the permeability of the BBB to radiolabelled GH, but not thyroid hormone stimulating hormone (TSH), luteinizing hormone (LH) or iodine. These findings suggest enhancement of an existing carrier rather than non-specific GH transport. Furthermore, the presence of receptors for GH on circumventricular organs of rats (Garcia-Aragon et al, 1992) and humans (Lai et al, 1991) indicate these receptors may be responsible for the passage of GH from the circulation to CNS structures. In addition, GH may enter the brain by retrograde blood flow along the pituitary stalk into the hypothalamus (Bergland et al, 1977; Bergland and Page, 1979; Sato et al, 1989), as high levels of pituitary hormones have been measured in the hypothalamic hypophysial blood (Paradisi et al, 1993). From here the hormones may reach the CSF by ependymal tanycytes or neuronal retrograde pathways (Jackson, 1984) for distribution to the brain.

In addition to pituitary origins, GH in the brain is also likely to reflect endogenous synthesis. This possibility is supported by the persistance of GH-ir in the amygdala of hypophysectomized rats (Pacold et al, 1978). Furthermore, Gossard et al (1987) using radiolabelled GH cDNA probes and ISH were able to show a wide distribution of GH mRNA in the brain. The distribution of GH mRNA did not, however, always correspond with sites of GH-ir. It is therefore possible that GH in the brain is derived from both neural and pituitary sites.

1.4.3. Characterization

The GH-ir material from the rodent brain shows similarities to its pituitary counterpart in terms of molecular weight estimates from chromatographic profiles (Hojvat et al, 1982a; Hojvat et al, 1982b). In addition, the biological effectiveness of brain GH appears to be equivalent to that of pituitary GH in stimulating tibial cartilage growth in hypophysectomised rats (Pacold et al, 1978; Hojvat et al, 1982b). Immunoadsorption studies, however, have indicated that the GH-ir is not rat GH but bears a close immunological similarity to the mid-portion of the hGH molecule (Lechan et al, 1981), suggesting some differences in conformation may exist between brain GH and native pituitary GH.

1.4.4. Regulation

Immunoassayable GH was demonstrated in the fetal rat brain at 10 days of gestation, prior to its appearance in the pituitary on day 12 (Hojvat et al, 1982a), indicating that the brain is an ectopic site for GH production. In addition, brain GH concentrations varied during ontogeny independently of pituitary GH levels. Growth hormone in fetal rat brain increases prior to parturition to reach adult levels. In contrast, pituitary GH increases postnatally to reach maximum concentration in the adult (Hojvat et al, 1982a). These observations indicate the presence of two biochemically and immunologically similar but topographically distinct and independent pools of GH, suggesting differences in the synthesis and regulation of brain and pituitary GH. The regulation of brain GH is, however, poorly understood.

Release of GH-ir from cultured amygdaloid cells was found to be autonomous unlike the release of GH from the pituitary gland which is under the control of hypothalamic releasing factors (Pacold et al, 1976; Pacold et al, 1977; Pacold et al, 1978). However, when SRIF was added to cultures of brain cells from amygdala, hippocampus or hypothalamus, the release of GH-ir was inhibited (Hojvat et al, 1982a). This inhibition was comparable to the effect of SRIF on pituitary function. Moreover, GH-ir fibres in the pre-optic hypothalamus of the sea lamprey were shown to be surrounded by SRIF-ir fibres (Wright, 1986), suggesting a possible physiological mechanism for regulation of brain GH by SRIF.

After hypophysectomy, an initial decrease in extractable brain GH was noted, however, 7 days post hypophysectomy there was an increase in GH-ir compared to intact animals (Hojvat et el, 1982b) suggesting an autoregulatory component in GH synthesis. In addition physiological situations leading to decreases in pituitary and serum GH, such as ovariectomy and thyroidectomy, were found to significantly increase GH-ir levels in the brain (Hojvat et al, 1986). These findings are compatible with the hypothesis that one function of brain-based GH is as a mediator of a short loop negative feedback system regulating the release of pituitary GH.

1.4.5. Actions of GH in the CNS

It is most probable that the role of GH within the CNS differs from the

currently ascribed endocrine activity of pituitary GH. This has been shown for many other peptide hormones which subserve many roles, some totally different from the effect for which they were named. Growth hormone receptor mRNA has been identified in the brain of many species including rats (Burton et al, 1992), guinea pigs (Harvey and Fraser, 1992), rabbits (Fraser et al, 1990) and chicken (Fraser et al, 1990). In addition, translation of the GH receptor occurs within the brain (Waters et al, 1990; Lobie et al, 1991) strongly suggesting CNS sites of action.

1.4.5.1. Brain Growth and Development

Studies have shown that the administration of purified GH to pregnant rats can produce a significant increase in brain weight and DNA content of their offspring. In addition, an increase in the density of cells associated with changes in the ratio of neurons to glia, and increases in the number and length of dendrites of cortical pyramidal cells has been noted (Zamenhof et al, 1966; Levi-Montalani and Angeletti, 1968; Sara et al, 1974). These actions appear to be selective for brain growth and because the placenta is believed to be impermeable to GH (Gitlin et al, 1965; Laron et al, 1966), these actions may be indirect and mediated by a somatomedin or similar trophic substance.

Growth hormone has previously been thought to be unimportant for post-natal maturation of brain. However, injecions of GH antiserum caused a retarded brain growth and decreased RNA and DNA synthesis (Pelton et al, 1974). A similar situation was observed in Snell dwarf mice and hydrocortisone intoxicated neonatal rats. In these animals, thymidine kinase, an enzyme associated with initiation of DNA synthesis and proliferation of glial cells and neurons, is strongly supressed throughout development (Noguchi et al, 1982a; Noguchi et al, 1982b). However, enzyme activity can be restored by exogenous GH (Noguchi et al, 1982a) and also by insulin-like growth factor-I (IGF-I) (Lenoir and Honegger, 1983). Bovine GH stimulates the endogenous activity of RNA polymerase I and II (Berti-Mattera et al, 1983) in hypothyroid neonatal rats. Moreover, incorporation of [^{3}H]-uridine into cerebral slices from these rats was also increased (Krawliec and Berti-Mattera, 1984), indicating a direct action of GH on RNA synthesis during post natal brain

development.

Bovine GH administered both intracisternally and intraperitoneally to neonatal rats significantly stimulates ornithine decarboxylase activity (Roger et al, 1974). Elevated activity of this enzyme correlates well with periods of maximum cell proliferation, stabilization of ribosome structure and promotion of RNA synthesis. Growth hormone may therefore regulate protein synthesis and growth via this enzyme. Ornithine decarboxylase activity is also increased in primary brain cell cultures (Yang et al, 1981; Noguchi et al, 1982d) and brain cell aggregates (Almazan et al, 1985) in response to bGH treatment. These actions appear to be dependent on thyroid hormone (Roger and Fellows, 1979) and this in turn may explain the abnormal brain development and impaired mental performance observed in hypothyroidism (Geel and Timras, 1970).

Pituitary deficient Snell dwarf mice have poor myelination due to decreased oligodendroglial proliferation (Noguchi et al, 1982c) in addition to reduced cell numbers in the brain (Viola-Magni, 1965; Winik & Grant, 1968; Reier et al, 1974; Sawchak, 1974). They have lower 2',3'-cyclic nucleotide 3'-phospohydrolase (CNPase) activity (an enzyme involved in myelinogenesis), which can be normalized with GH treatment (Noguchi et al, 1982d), possibly through enhancement of glial cell division. The CNPase activity *in vitro* is also increased by GH, as is myelin basic protein (Almazan et al, 1985). Thus, GH may be necessary for normal myelinogenesis in brain development.

Morphological studies of the somatosensory area of the rat cerebrum after GH antiserum injection demonstrated an abnormal accumulation of undifferentiated glial cells (Pelton et al, 1974). This may result from arrested migration of the glial cells from the subependymal zone, and this was also associated with decreased amounts of stainable myelin. These results suggest the decreased myelin formation seen in GH deficiency may be attributable to a failure of glial precursors to migrate from the subependymal zone and subsequently differentiate into oligodendrocytes (Pelton et al, 1977).

1.4.5.2. Neurotransmission

Growth hormone may play a role in neurotransmission by affecting many of the classical pathways. Rat GH has been shown to reduce catecholamine levels in the median eminence (ME). A rapid reduction in dopamine (DA) turnover in the external layer of the ME in hypophysectomised rats (Andersson et al, 1977), along with decreased nor-adrenaline (NA) levels in the subependymal layer and decreased NA turnover in the posterior periventricular hypothalamus (Andersson et al, 1983) has been observed. Expression of the bGH gene in transgenic mice is also associated with a reduced turnover rate of DA and NA in different regions of the hypothalamus (Steger et al, 1991). However, mice transgenic for hGH-N and hGH-V genes had increased neurotransmitter levels probably due to an action on lactogenic receptors.

Snell and Ames dwarf mice are deficient in both DA and its metabolites in the ME and striatum, and overall catecholamine biosynthesis is suppressed (Kempf et al, 1985; Morgan & King, 1986). Growth hormone treatment also lowers the levels of homovanillic acid, a DA metabolite in GH deficient adults (Burman et al, 1993), suggesting prolonged DA transmission. These results seem to indicate that GH may exert acute short loop feedback actions in part by reducing DA synthesis and turnover in discrete regions of the brain, including the DA terminals inhibiting SRIF release in the ME. Growth hormone has also been shown to decrease GRF synthesis and release from rat hypothalamus (DeGennaro-Colonna et al, 1988; Fernandez-Vasquez et al, 1993). The reduced NA synthesis and turnover caused by GH may lead to this reduced secretion of GRF via actions on α-adrenoceptors in the hypothalamus (Eden et al, 1981; Andersson et al, 1983). However, NA acting at β-adrenoceptors may also act to inhibit GH secretion. The affinity of β-adrenoceptors is significantly increased by GH treatment although the number of binding sites does not change (Popova et al, 1991b), and this may compensate for the reduced NA levels accompanying GH treatment. It is assumed that these β-adrenoceptors mediate an effect stimulatory to SRIF and therefore inhibitory to GH secretion (Popova et al, 1991b).

Serotonin (5-HT) is thought to be a stimulatory transmitter in the regulation of GH release, probably acting through inhibition of SRIF (Arnold and Fernstrom, 1980). Blockade of 5-HT receptors with methysergide or cyproheptadine was shown

to supress GH secretion (Stuart et al, 1976), and conversely GH was demonstrated to reduce 5-HT synthesis in the ME, mediobasal hypothalamus (MBH), diencephalon and pons-medulla (Stern et al, 1975b; Morgan and King, 1986). In addition, GH decreased 5-HT binding capacity and receptor affinity in both hypothalamus and pituitary but not cerebral cortex (Popova et al, 1991a). Growth hormone deficiency in Snell dwarf mice is also accompanied by enhanced 5-HT levels in the striatum and hippocampus (Morgan and King, 1986). Similarly, elevated levels of tryptophan (the 5-HT precursor) were detected in the whole brain of hypophysectomized and dwarf mice (Cocchi et al, 1975a; Cocchi et al, 1975b). These studies also seem to indicate that 5-HT - GH interactions may play a role in GH autoregulation.

Growth hormone may also affect synthesis of acetylcholine (ACh), as Snell dwarf mice displayed both diminished choline acetyltransferase activity and choline uptake in hippocampus, olfactory tubules and striatum (Fuhrmann et al, 1985). In addition these deficiencies could be corrected with GH treatment (Fuhrmann et al, 1986).

1.4.5.3. Motor Activity

Snell dwarf mice have impaired levels of spontaneous locomotion compared to normally growing mice (Noguchi et al, 1982a; Fuhrmann et al, 1985), which can be normalized with GH treatment (Noguchi et al, 1982a). Diminished motor activity may be attributable to the deficiencies in DA/5-HT balance in the nigrostriatal tract (Kempf et al, 1985), and alterations in cholinergic transmission in the basal ganglia (Fuhrmann et al, 1985). The decreased myelin formation associated with GH deficiency (Pelton et al, 1977; Noguchi et al, 1982c) may also account for this impaired motor development.

1.4.5.4. Sleep

A role for GH in sleep has been suggested based on observations in patients with GH disturbances (deficiency or excess) who have abnormal sleep patterns compared with normal subjects. Young adults displaying isolated GH deficiency (IGHD) have reduced deep sleep (slow wave sleep (SWS)), whereas total sleep time

(TST) and non rapid eye movement (REM) sleep are increased (Guilhame et al, 1982; Astrom and Lindholm, 1990). Long term administration of synthetic GH or normalization of GH levels was able to restore these sleep patterns towards normal (Guilhame et al, 1982; Astrom et al, 1990).

Acromegalics with a hypersecretion of GH, have abnormal sleep patterns characterized by decreased REM sleep, more night time awakenings, increased daytime sleepiness, low to normal TST and decreased SWS (Astrom et al, 1990; Grunstein et al, 1991). However, the energy of the SWS and REM sleep (related to underlying neuronal activity in the cerebral cortex) was much higher in patients with elevated plasma GH than in the same patients after normalization of plasma GH by adenomectomy (Astrom and Trojaborg, 1992). A large percentage of acromegalics also suffer from sleep apnea which is related to the degree of GH hypersecretion (Grunstein et al, 1991). Growth hormone receptors are present in the hypothalamus, the area of the brain which controls breathing, thus control of breathing during sleep maybe related to GH secretion (Fraser et al, 1990; Hasegawa et al, 1993).

Growth hormone may also control some sleep parameters in normal subjects. Exogenous GH increased REM sleep but had no effect on SWS in rats (Drucker-Colin et al, 1975) or cats (Stern et al, 1973; Stern et al, 1974). This effect may be mediated by changes in protein synthesis as GH counteracted the inhibitory effects of Asinomycin on both REM sleep and protein synthesis (Drucker-Colin et al, 1975).

1.4.5.5. Learning and Memory

The possibility that increased brain development induced by GH may enhance learning was suggested by Zamenhof in 1942. The offspring of pregnant rats given pGH were found to have increased brain weight and cellular content with an increase in the number of neurons in the supragranular layers of the cortex (Zamenhof et al, 1942; Zamenhof et al, 1966; Sara and Lazarus, 1974). At maturity these rats also displayed a superior learning ability. However, since GH delays parturition and alters maternal behaviour, increased learning may be a result of prolonged gestation or increased frequency and duration of nursing (Croskerry et al, 1973). The GH deficient state induced by hydrocortisone intoxication is also associated with diminished learning

ability which can be corrected with exogenous GH (Noguchi et al, 1982a), suggesting the impaired learning is specifically caused by GH deficiency. A similar situation is encountered with Snell dwarf mice which are GH deficient (Bouchon and Will, 1983). Lower intelligence quotient (I.Q.) scores, decreased head circumference (indicating reduced brain development), and deficiencies in visuomotor functioning have also been observed in children with GH deficiency (Laron and Galatzer, 1981; Laron and Galatzer, 1985). These characteristics could be improved with GH treatment, especially if treatment began before age 5 (Laron and Galatzer, 1985), which may indicate that a critical age at which GH affects brain development and maturation exists.

Growth hormone has also been implicated in alterations of long term memory. Stern and Mogane (1977) suggested that GH may act to retrospectively enhance long term memory during sleep, as learning was enhanced when followed by sleep. The sleep induced GH surge stimulating REM sleep and protein synthesis may facilitate establishment of these new memories (Drucker-Colin et al, 1975; Mendelson et al, 1980). In contrast, Ekstrand et al (1977) speculated that GH has a proactive inhibitory effect on human memory. Subjects who were aroused from sleep and asked to learn and later recall verbal material showed significantly more forgetfulness than subjects who remained awake. In addition, Hoddes (1979) indicated that GH selectively induces suppression of memory when GH is administered in close proximity to learning in a maze test. However, as the time lapse between GH injection and learning the task was increased, the inhibitory effect on memory was reversed. These results may indicate GH has an inhibitory effect on very short term memory whereas it may enhance long term memory.

1.4.5.6. Feeding

Growth hormone has been reported to promote food intake in several species including reptiles and birds. Bovine GH injected into juvenile lizards and toads increased food intake by 300 and 100% respectively (Licht and Hoyer, 1968; Zipser et al, 1969). In addition exogenous GH was found to counteract the hypophysectomy induced weight loss in pigeons and increase their food intake (Bates et al, 1962).

Buntin and Figge (1989) reported a similar increase in food intake in ring doves receiving intracerebroventricular (i.c.v.) injections of tGH, oGH, and hGH. The ventromedial nucleus and lateral region of the hypothalamus have been implicated in the control of feeding in many species (Bray et al, 1985). Growth hormone immunoreactivity has also been demonstrated in these regions (Lechan et al, 1983), lending support to the hypothesis that GH may have direct effects on control of food intake. However, GH may also protect against excessive food consumption via an indirect action on IGF-I levels. Preparations rich in IGF-I have been demonstrated to cause diminution in body weight associated with a decrease in food intake (Tannenbaum et al, 1983), whereas insulin-like growth factor-II (IGF-II) does not appear to alter food intake (Harel and Tannenbaum, 1992). In addition, somatomedin levels are generally depressed in undernutrition (Phillips and Vassiloupoulou-Sellin, 1979).

1.5. IMMUNE GROWTH HORMONE

The neuroendocrine and immune systems are now recognised to be intimately linked and involved in bi-directional communication (Weigent and Blalock, 1987; Blalock, 1989; Kelley, 1989; Gala, 1991; Gilbert and Payan, 1991; Kelley, 1991; Kelley et al, 1992), with neuroendocrine hormones having immunoregulatory effects and cytokines influencing neuroendocrine function. Growth hormone is thought to play an important role in regulation of these systems and has been shown to exert its actions on many aspects of neuroendocrine and immune function.

1.5.1. General GH Actions

Initial data implicating GH as a modulator of the immune system came from two sources, a) injection of GH antiserum into normal mice resulted in thymic atrophy (Pierpaoli and Sorkin, 1968) and suppression of immune function (Pierpaoli et al, 1969) and b) Snell dwarf mice with a congenital deficiency in GH production, possess depressed immune responses and involuted central and peripheral lymphatic tissues, along with cellular depletion of the bone marrow (Baroni et al, 1967). The thymus gland is especially affected in this species, and pituitary deficient rats and

dogs also possess small thymus glands (Roth et al, 1984). These observations along with the lack of an effect of GH in thymectomized mice (Fabris et al, 1971), and the nude rat which lacks a thymus (Davila et al, 1987), suggest that this gland may be an important site for GH action. In addition, the concentration of the thymic hormone thymulin, is reduced in the plasma of dwarf mice (Pelletier et al, 1976) and this reduction may accompany the thymic involution known to occur with ageing. Low thymulin levels are also observed in GH-deficient children (Mocchegiani et al, 1992), whereas middle aged acromegalic patients were found to have elevated thymulin serum concentration (Mocchegiani et al, 1992; Timsit et al, 1992) which positively correlated with the level of IGF-I but not GH in the serum.

These observations led to studies aimed at normalizing immune function with GH treatment. Implantation of GH_3 pituitary cells (which produce GH and PRL) into aged rats, restored the deteriorated thymic structure (Kelley et al, 1986) and GH administration reversed thymic atrophy in dwarf mice (Pierpaoli et al, 1969). Injections of bGH to aged dogs (Goff et al, 1987), oGH to mice and rats (Goya et al, 1992; Goya et al, 1993), and hGH to GH-deficient children (Mocchegiani et al, 1990) was able to partially restore low serum thymulin levels. It therefore seems that GH augments synthesis of thymulin by the thymic epithelial cells and this hormone is known to play an important role in regulation of the immune system by stimulating release of cytokines such as interleukin-2 (IL-2) and enhancing T-cell mediated cytotoxic responses.

1.5.2. Bidirectional Communication

Not only does GH affect the immune system but several factors thought to be involved in the immune function have been found to exert profound effects on the pituitary. Exposure to bacterial endotoxin has long been shown to stimulate the release of anterior pituitary hormones. Endotoxin is a potent stimulator for production of endogenous pyrogenic protein interleukin-1 (IL-1) by macophages and monocytes. Bernton et al (1987) showed that IL-1 was capable of initiating secretion of GH by pituitary cells both *in vitro* and *in vivo*, suggesting that IL-1 may act directly on the pituitary cells to modulate GH secretion. Both IL-1 and IL-2 administration to rats

results in an increase in blood adrenocorticotrophic hormone (ACTH) and corticosterone levels (Lotze et al, 1985; Besedovsky et al, 1986), thus implying that common elements can be percieved by both systems. These data indicate that the process of infection and inflammation and consequent immune activation may serve as a potent pituitary stimulus for secretion of GH and other pituitary hormones, which may then play a role in the complex events of the immune response.

1.5.3. Direct Actions of GH on Cells Involved with Immunity

A number of authors have described the presence of GH binding sites on both thymic and lymphoid cells and the lymphocyte cell line IM-9 (Arrenbrecht, 1974; Lesniak et al, 1974; Stewart et al, 1983; Kiess & Butenandt, 1985; Ban et al, 1991; Yang et al, 1993). However, the distribution of these receptors on specific subsets of immune cells, such as T- and B-lymphocytes and Natural Killer cells (NK) is unknown. Human lymphoid cells appear to have a high affinity receptor for hGH with approximately 7000 hGH binding sites per cell, whilst the IM-9 cell line has 4000 binding sites per cell. The presence of these binding sites provide a natural mechanism for transducing information from the binding of GH on the surface membrane to the cytoplasm of lymphoid cells.

1.5.3.1. GH and Lymphocytes

Growth hormone has been demonstrated to have profound influences on both the development of the immune system as a whole, and the development of T-cell dependent immune responses in particular (Snow, 1985). The thymus is the endocrine organ in which bone marrow derived precursors proliferate and differentate into mature T-lymphocytes by interacting with the thymic microenvironment. Growth hormone injections affect thymocyte progenitor cells at the early stages of their development, by potentiating the effects of granulocyte/macrophage colony stimulating factor (GM-CSF). This leads to an increase in both cell numbers and the percentage of granulocyte cells (Knyszynski et al, 1992). In addition, GH_3 cell implants also increase the percentage of CD4+ and CD8+ T-lymphocytes in the thymus of ageing rats (Li et al, 1992). Recombinant cGH also increases these cell numbers in obese

strain chickens (Marsh, 1992; Marsh et al, 1992). These effects may be the result of a direct GH action. However, antibodies against the IGF-I receptor were found to block the enhanced cell numbers caused by GH and no increases in cell number were seen following *in vitro* stimulation, suggesting that this mechanism involves stimulation of IGF-I production. GH treatment *in vivo* also causes proliferation of thymocytes in both hypophysectomized rats (Berczi et al, 1991) and aged mice (Goya et al, 1992). Cell populations in secondary lymphoid organs may also be stimulated by GH. The activation of human T-lymphocytes by recombinant hGH results in an increased ability of these cells to traffic to, and engraft in the spleen of severe combined immune deficiency (SCID) mice which normaly have no endogenous lymphocytes (Murphy et al, 1992). Growth hormone induces the proliferation of human T-lymphoblasts and again this effect appears to be mediated by IGF-I (Geffner et al, 1990; Gala and Shevach, 1993). Improved wound healing (Lynch et al, 1989; Steefos and Jansson, 1992) and reduced tumour metastasis (Torosian and Donoway, 1991) have also been reported as positive effects of GH. Growth hormone augments the activity of cytotoxic T-lymphocytes *in vitro* (Kelley, 1991). Ageing is associated with a hyposecretion of GH and decreases in natural immunity provided by the cytolytic NK cells which release interferon (IFN). A deficiency of GH has been associated with impairment of NK cell activity in both experimental animals and humans (Kiess, et al, 1986; Crist et al, 1987). There is also a decline in NK cell activity with age and this decline can be partially corrected with GH therapy in women (Crist et al, 1987) and aged mice (Davila et al, 1987). In addition to T-cells, both GH and IGF-I enhance immunoglobulin (Ig) production and proliferation in human B-cell lines (Kimata and Yoshida, 1994), indicating that GH may also function as a B-cell stimulatory cytokine.

1.5.3.2. GH and Phagocytic Cells

Phagocytosis and subsequent destruction of invading microorganisms by macrophages is considered to be the first line of defence of host against bacterial and parasitic infection. The classic macrophage activating factor is IFNγ, but recently GH has been demonstrated to prime alveolar macrophages *in vitro* when these cells are

triggered with opsinized yeast particles (Edwards et al, 1988; Edwards et al, 1992). These myeloid cells can be triggered to produce reactive oxygen intermediates that non-specifically kill ingested organisms. *In vivo* administration of either rGH or pGH to hypophysectomised rats was able to stimulate increased superoxide anion (O_2^-) production by macrophages with a similar potency to injections of IFNγ (Kelley, 1991). In addition, GH increased the resistance of hypophysectomised rats to *Salmonella typhimurium*, probably by increased activity of macrophages to secrete O_2^- (Edwards et al, 1992). Macrophages can also be activated to kill tumour cells by secretion of tumour necrosis factor (TNFα). Hypophysectomy caused an 80% reduction in the amount of TNFα secreted from rat peritoneal macrophages triggered *in vitro* with lipopolysaccharide (Kelley, 1991). In addition, this effect could be almost completely reversed by injections of pGH or rGH and matched the effects produced by IFNγ in priming macrophages for TNFα production.

Growth hormone has a similar effect on polymorphonuclear phagocytes (neutrophils), to increase both resting and stimulated levels of reactive oxygen intermediates in GH deficient children (Rovensky et al, 1982), as well as neutrophils from normal and aged subjects (Wiedermann et al, 1991a; Wiedermann et al, 1991b). Acromegalics were also observed to have higher resting and antigen elicited O_2^- production. It appears that the hPRL receptor may account for the hGH mediated priming of neutrophils for an enhanced respiratory burst (Fu et al, 1992). However, neither pGH or bGH bind to the PRL receptor, yet they prime porcine and bovine neutrophils respectively for O_2^- secretion, suggesting that the GH receptor mediates this action in some species.

Migration of monocytes into different tissues appears to occur randomly, however, at sites of inflammation monocytes accumulate by migrating along gradients of chemoattractants released by damaged tisssue. Exogenous administration of recombinant hGH was found to correlate with an enhanced random migration of circulating monocytes both *in vivo* and *in vitro* (Wiedermann et al, 1992). Later experiments by this group showed recombinant hGH was a potent chemoattractant for monocytes, whose effects could be antagonised by SRIF (Wiedermann et al, 1993). Thus, GH may play a role in directing monocytes into tissues where they can be

transformed into macrophages at sites of contact with the external environment.

1.5.4. Production of GH by Immune Cells

Numerous studies have now established that cells of the immune system have the ability to synthesize and secrete neuroendocrine hormones (Weigent and Blalock, 1987; Blalock, 1989), although this expression may be of a transient nature and occur only in neonates (Binder et al, 1994). Using slot blot hybridization and Northern analysis, Weigent et al (1988) were able to demonstrate the presence of a GH mRNA species in spleen, thymus, and bone marrow leukocytes from both rats and humans. These cells produced an ir-GH *de novo*, which appeared to be closely related to pituitary GH in terms of antigenicity, molecular size and bioactivity (Weigent et al, 1987; Weigent et al, 1988; Weigent et al, 1990; Hattori et al, 1990; Varma et al, 1993). In addition, these authors found multiple forms of ir-GH which may represent oligomers or GH linked to plasma proteins. Although some studies have indicated the sequence of GH mRNA (Kao et al, 1992) and the protein (Baglia et al, 1992), produced from immune cell lines may be different from pituitary GH, the GH mRNA from rat and human lymphocytes was structurally similar to pituitary GH based on Southern blot and restriction endonuclease digestion analysis (Weigent and Blalock, 1991; Weigent et al, 1991a). Therefore any differences in protein may be due to translational or post-translational events. Message levels were stimulated after Freunds complete adjuvant (Weigent et al, 1991b) and phytohaemaglutinin (PHA) antigen (Baxter et al, 1991) treatment, indicating GH is released in response to antigenic challenge. Furthermore, immune cell proliferation (incorporation of [^{3}H] thymidine) and synthesis of GH was inhibited when cells were incubated with an antisense oligonucleotide to GH mRNA (Weigent et al, 1991c), indicating a role for ir-GH in cell replication. Primitive invertebrates such as *Viviparus ater* contain GH-ir in hemocytes (Ottaviani et al, 1992) which are the ancestral cell responsible for defense mechanism against infection in these animals. Therefore, it appears that GH is present in the immune system across a wide variety of species.

Although the GH molecule produced by leukocytes appears to be substantially similar to pituitary GH, the mechanisms regulating the synthesis and secretion of this

protein appear to share similarities and dissimilarities with the mechanisms operating at the level of the pituitary. Thus, a GRF-like peptide and its mRNA are produced by immune cells (Stefanou et al, 1991; Weigent et al, 1991a). This peptide along with hypothalamic GRF induce GH mRNA transcription in both pituitary cells and lymphocytes (Weigent et al, 1990), indicating a similar stimulatory mechanism to that seen in pituitary gland. However, even though SRIF is localized and synthesized in lymphoid organs (Aguila et al, 1991) it does not appear to inhibit GH production by lymphocytes (Hattori et al, 1990). Growth hormone may also unusually, upregulate its own secretion (Hattori et al, 1990; Hattori et al, 1993). The same cells that produce GH also synthesize IGF-I (Weigent et al, 1992), and production of this growth factor can be inhibited by antibodies specific for GH. In addition, IGF-I has been shown to decrease the level of GH mRNA and protein (Baxter et al, 1989; Baxter et al, 1991) in rat spleen cells, but not in mitogen stimulated human lymphocytes (Hattori et al, 1993). The role of IGF-I in feedback regulation of GH may therefore be important under resting conditions but not during the immune response when recruitment of cells to areas of infection is important. Several other neuropeptides, monoamines and regulators of GH gene expression, including TRH, NA and estrogen did not alter ir-GH secretion from lymphoid cell lines, although dexamethasone was found to inhibit ir-GH secretion (Kao and Meyer, 1992). As glucocorticoids are known to act as transactivators and bind to the GH gene promoter, this may indicate some homology between pituitary and immune GH gene promoters. In addition, Pit-1/GHF-1 gene expression has been demonstrated in lymphoid tissues (Delhase et al, 1993) and this important pituitary transcription factor may play a role in the control of GH gene expression in immune cells.

Like many of the interleukins, GH has been shown to exhibit a number of pleiotropic actions on cells of the immune system. Growth hormone can regenerate thymic tissue in aged subjects and augment differentiation of precursor cells into mature lymphocytes. Growth hormone causes proliferation of cells involved in immunity and is as potent as IFNγ in priming macrophages for production of O_2^-. Growth hormone is also synthesized locally by leukocytes which implies it may act locally as a cytokine in lymphoid tissues and the presence of GH receptors on immune

cells suggests autocrine or paracrine roles for GH in the immune system.

1.6. PLACENTAL GROWTH HORMONE

The existence of a gene coding for a GH variant (hGH-V) was first appreciated when extensive sequence analysis of the hGH gene cluster demonstrated a gene that differed from the authentic GH gene by only 34 base substitutions, and potentially encoded a protein product that differed from pituitary GH by 13 amino acid residues (Seeburg, 1982). Although no protein corresponding to this gene had been identified, the gene itself displayed all the characteristics attributable to an expressed gene and it was found that it could be expressed in established cell lines (Pavlakis et al, 1981). Subsequently hGH-V mRNA was identified (Frankenne et al, 1987) and localized to the syncytiotrophoblastic epithelium of the placental villi (Cooke et al, 1988a; Liebhaber et al, 1989). A 22 kDa GH-like antigen had been described previously by Hennen et al (1985), which was suggested to be of placental origin. Appearance of this GH-like antigen in blood was accompanied by a simultaneous loss of pituitary GH secretion after the 25th week of pregnancy. The levels increased up to term, and rapidly disappeared upon delivery. Messenger RNA levels also increased in concentration throughout pregnancy (Soares et al, 1991; Golos et al, 1993).

1.6.1. Splice Variants

The hGH-N and the closely related hGH-V genes differ in their patterns of splice site selections. The hGH-V transcript does not utilize the same splicing pathway as hGH-N due to 3 base differences located between the two potential splice acceptor sites (Estes et al, 1990). However, a hGH-V2 transcript has been identified in placenta (Cooke et al, 1988a; Cooke et al, 1988b) which accounts for approximately 5-7% of the hGH-V mRNA in the first trimester and 15% at term. This variant retains intron D (MacLeod et al, 1992), and may encode a 26 kDa protein (Liebhaber et al, 1989). A similar phenomenon has been observed with the bGH transcript which sometimes retains intron D as a result of a 35 kDa *trans*-acting factor that binds *cis*-sequences or exonic splicing enhancers located in exon 5 (Sun et al, 1993; Dirksen et al, 1994).

1.6.2. Differential Expression of the hGH-V Gene

Several mechanisms appear to play a role in the tissue specific regulation of the GH genes. DNase 1 hypersensitivity sites in chromatin often signal the presence of *cis*-acting elements involved in the control of gene transcription (Gross and Garrad, 1988). A complex pattern of these sites is distributed along the hGH locus, most of which appear to be restricted to cells of placental origin. Placental specific hypersensitive sites within the first intron of the hGH-N and hGH-V genes have been identified (Jimenez et al, 1993). It has been suggested that extensive alteration in chromatin structure of the hGH locus may therefore be required for transcriptional activation and control in placental cells.

In contrast to the hGH-N gene, hGH-V appears to have only a single site for Pit-1/GHF-1 binding. This is the distal site described in hGH-N, located -107/-140 bases upstream of the mRNA start site (Nickel et al, 1991b). This distal binding site has only a single base difference compared to hGH-N, whereas the proximal Pit-1/GHF-1 site contains 7 base changes, which clearly prevents efficient Pit-1/GHF-1 binding. Other members of the placental GH family do not share these sequence changes. CS shares sequence homology with hGH-N at its proximal Pit-1/GHF-1 binding site, and Pit-1/GHF-1 has been shown to bind to this region but not the distal region in this CS gene (Nickel et al, 1990). In addition, CS genes may contain a transcriptional enhancer downstream from the structural gene (Rogers et al, 1986). These data indicate that the pattern of binding is distinct for the placental members of the GH family and this may also be related to a different temporal pattern of expression of the genes in the developing trophoblast (Nickel et al, 1993). The hGH-V gene may also contain a T_3 responsive region, as this hormone has been shown to increase hGH-V mRNA levels in placental carcinoma cells (Nickel et al, 1991a), but not in pituitary tumour cells (Nickel and Cattini, 1992).

1.6.3. Pituitary Specific Repression of the Placental GH Family

Negative regulation is implicated in the transcriptional control of several genes including hGH-N (Treacy et al, 1991). Highly conserved regions in the distal 5' flanking sequences of the CS and hGH-V genes but not the hGH-N gene have been

identified. These DNA elements located in a 263 bp fragment about 2kb upstream of the genes are able to repress PL promoter activity >98% in pituitary cells after gene transfer. However, they permit efficient promoter activity in placental cells (Nachtigal et al, 1993a; Nachtigal et al, 1993b). These sequences (PSF-A and PSF-B) appear to bind a single protein factor (pituitary specific factor), which is probably absent from placenta but appears to be otherwise ubiquitous. This mechanism may therefore play a role in tissue specific expression/ repression of GH-V, however, recently the GH-V gene has been shown to be weakly expressed in the pituitary (Scrippo et al, 1991) indicating other factors may play a more important role in expression.

1.6.4. GH Synthesis and Regulation in Pregnancy

Maximal expression of the placental GH genes occurs between 12 and 20 weeks of gestation and plateaus through term (Golos et al, 1993). In normal men and non-pregnant women GH is secreted in episodic bursts with low or undetectable levels between peaks (Zadik et al, 1985; Ho et al, 1987). This pulsatile pattern was totally abolished after 15-17 weeks of gestation until term in pregnant women, and was replaced by a continous stable secretion (Eriksson et al, 1988; Eriksson et al, 1989).

Previous studies have shown that both rat and mouse placenta are sites of GRF synthesis (Meigan et al, 1988; Suhr et al, 1989; Margoris et al, 1990) and it is possible that hGH-V may be a target for this placental GRF. However, de-Zeghler et al (1990) showed that Serorelin (GRF-(1-29)-NH_2) a GRF analogue did not affect hGH-V secretion, and pituitary GH was also unresponsive to this hypothalamic hormone during pregnancy. Insulin-like growth factor-I is a highly potent inhibitor of somatotrophs (Yamashita et al, 1986) and is likely to be responsible for the inhibition of the maternal pituitary GH during pregnancy. The levels of IGF-I progressively increase up to term (Wilson et al, 1982), and this effect has been shown to be independent of the pituitary gland (Hall et al, 1984). Evidence that hGH-V is the main stimulator of IGF-I in pregnancy was provided by Beckers et al (1990). These authors demonstrated that the pulsatile pituitary GH secretion persisted during pregnancy in acromegalic women. However, an increase in levels of IGF-I was

observed when the patients were pregnant compared to when they were not. Although the pituitary gland is essental for fertility its role seems only minor in the maintenance of pregnancy. The findings of the studies cited indicate the complexity of the placental hormonal milieu and its regulation during pregnancy. It also seems that although hGH-V is growth promoting in mice it may not perform the same function in humans, as individuals lacking hGH-N are not compensated by possessing hGH-V.

1.6.5. Roles of hGH-V

The physiological role of the hGH-V protein is not known, but is likely to be similar to that of pituitary GH and PL, resulting in repartitioning of nutrients in the fetal-placenta unit. The possibility that hGH-V protein acts as a regulator of maternal carbohydrate and lipid metabolism was suggested by Goodman et al (1991). These authors demonstrated the ability of hGH-V protein to increase glucose oxidation (an insulin like response), induce refractoriness to insulin stimulation, and reduce lipolysis in the presence of glucocorticoids. It was therefore concluded that placentally expressed hGH-V has a spectrum of activity comparable to pituitary GH. Unlike pituitary GH secretion, the placental GH is essentially secreted in a continous fashion (Eriksson et al, 1989) which would have profound effects on a variety of metabolic functions, again indicating importance in regulation of metabolism during pregnancy.

At present hGH-V protein appears to be a hormone unique to humans. It behaves as a complete agonist to pituitary GH as shown in radioligand receptor assay (Hennen et al, 1985). Although hGH-N and hGH-V proteins possess a highly homologous primary structure differing by only 13 amino acids (Cooke et al, 1988a), ICC, isoelctric focusing and gel filtration results suggest that the two proteins have significantly different tertiary and quaternary structures (Selden et al, 1988). The hGH-V protein also appears to be more basic than pituitary 22 kDa and 20 kDa GH (Frankenne et al, 1988).

Several authors have characterized the binding charactristics of hGH-V protein compared to hGH-N protein. The hGH-V protein was found to displace both PRL and GH binding to liver microsomes (Ray et al, 1990), indicating a dual specificity previously thought to be unique to hGH-N. This finding was supported by both

Nickel et al (1990) and MacLeod et al (1991), but they indicated a greater selectivity for binding to somatogenic as opposed to lactogenic receptors when compared with hGH-N protein. This may explain the growth promoting activity of the variant protein in transgenic mice (Selden et al, 1988), and the lower lactogenic activity as compared to pituitary GH (Igout et al, 1992). Human GH-V was also found to be equipotent to hGH-N as a ligand for the GH binding protein (Baumann et al, 1991) and presence of high levels of GH receptor/binding protein immunoreactivity in the placenta (Ymer et al, 1989; Garcia-Aragon et al, 1992) implies that hGH-V may play a role in prenatal development.

1.7. PINEAL GROWTH HORMONE

The pineal gland is thought to be involved in the interactions between adenohypophyseal hormones and the neuronal activity of the hypothalamus. Using antisera specific for protein sequences in GH, Noteborn et al (1993) detected immunogenically related regions in proteins extracted from ovine pineal tissue. These authors also found a heterogeneity with respect to molecular size, similar to that observed with pituitary GH. The detection of GH in the pineal gland has led to the speculation that this protein may function as a neuromodulator or neurotransmitter in this tissue.

1.8. MAMMARY GROWTH HORMONE

GH has recently been reported to be produced in the dog mammary gland (Selman et al, 1994). In this species, progestin induces GH excess, which is present even after hypophysectomy. However, normal levels of circulating GH are observed after removal of the mammary gland. High levels of GH-ir have been demonstrated in this tissue, primarily in hyperplastic ductular epithelial elements and neoplastic epithelium of mammary tumours. These data therefore indicate that the GH is produced at an extrapituitary site, and may be involved in mammary tumorigenesis.

1.9. OTHER TISSUES

Studies by Kaganowicz et al (1979) indicated the presence of an ir-GH like

peptide in normal human ovary, and presence of ir-GH has also been reported in liver, kidney, lung, muscle, colon, and stomach (Kyle et al, 1981). However, the presence of GH-like material in these tissues does not necessarily indicate synthesis by the cells themselves, and may constitute contamination by blood in the tissues which would for example contain lymphoid cells capable of GH synthesis. Another possibility is that the hGH-like material is bound specifically to GH receptors on the cell surface, where it may take several hours to dissociate.

1.10. EXPERIMENTAL RATIONALE

Many reports have appeared on the presence of ir-GH in extra-pituitary sites. While this does not necessarily indicate synthesis in these tissues, some studies have provided evidence to suggest that a GH gene is expressed. These peptides may be the product of as yet unidentified genes, and sequence differences may provide evidence that GH plays different roles in these ectopic tissues. The ability of one hormone to direct multiple effects have mostly focused on the existence of specific subtypes of receptors, or existence of multiple active domains within a hormone. However, the wide array of functions of a hormone may in part be attributable to its range of variants. The heterogeneity of GH is already well established and is due the presence of multiple genes, post-transcriptional processing, and post-translational modifications (Baumann, 1991). Thus, the discovery of a GH-like peptide in an extra-pituitary site may add to this GH heterogeneity.

Little is known about these ectopic GH species at the molecular level. Although extra-pituitary GH has been documented clinically and in rats, ectopic sites of GH production or localization have yet to be demonstrated in other species. It may, therefore, not be a common phenomena, and may be a late evolutionary development. The possibility that extra-pituitary sources of GH may be present in a lower vertebrate, the domestic fowl, was therefore examined in this thesis. Growth hormone chemistry, regulation and roles in the chicken has been extensively examined (Harvey et al, 1991; Scanes et al, 1993), which would allow comparisons between pituitary GH and extra-pituitary GH to be made. Several unique characteristics of GH physiology also suggest the chicken would be a useful model to study ectopic GH

production.

Circulating GH levels in this species are several orders of magnitude higher than in mammals (Scanes et al, 1983), and this may facilitate detection of ectopic GH by immunological and molecular techniques. This hypersecretion of GH has resulted in the chicken being proposed as a model to study acromegaly, a pathological state in humans which is often characterized by ectopic GH production by tumourous tissue (Harvey, 1987). Growth hormone, moreover, is measurable at low concentration in the plasma of hypophysectomized chickens, suggestive of an ectopic site of GH production (Harvey et al, 1987; Lazarus & Scanes, 1988).

1.11. HYPOTHESES

The specific hypotheses tested in this thesis are:

1. GH gene expression occurs ectopically in central and peripheral tissues of the domestic fowl.
2. The GH gene ectopically expressed is identical to that expressed in the pituitary gland.
3. The GH gene is translated to immunoreactive proteins in these ectopic tissues.

1.12. TABLES

Table 1.1. - Possible Sources of hGH Heterogeneity

Source		Reference
A. Genome	2 genes (hGH-N and hGH-V)	Seeburg, 1982
B. Transcriptional events	2 alternative promoters?	Courtois et al, 1992
C. Post-transcriptional events	2 mature mRNAs (22 KDa and 20 KDa	Lewis et al, 1980
	17.5 kDa?)	Lecomte et al, 1987
D. Precursor molecule	Pre-hGH	Seeburg, 1982
E. Post-translational events	Deamidation	Lewis et al, 1979; 1981
	Acetylation	Lewis et al, 1979
	Glycosylation (hGH-N?)	Sinha et al, 1986; Diaz et al, 1993
	(hGH-V)	Ray et al, 1989
	Proteolysis (2-chain GH)	Singh et al, 1974
	Aggregation (Oligomers)	Lewis et al, 1977; Stolar et al, 1984
	Phosphorylation	Baldwin et al, 1983
F. Postsecretory events	Complexing with plasma binding proteins	Baumann et al, 1991
G. Metabolic conversions	Fragments (GH_{1-43})	Singh et al, 1983; Nicoll et al, 1986

Table 1.2. - Chicken GH Heterogeneity

Source		Reference
A. Genome	One gene	Tanaka et al, 1992
B. Transcriptional events	?	
C. Post-transcriptional events	?	
D. Precursor molecule	Pre-cGH	Lamb et al, 1988
E. Post-translational events	Deamidation ?	
	Acetylation ?	
	Glycosylation	Berghman et al,1987; Aramburo et al, 1991
	Proteolysis ?	
	Aggregation (Oligomers)	Aramburo et al,1990b
	Phosphorylation	Aramburo etal,1989a; Aramburo etal,1990a; Aramburo et al, 1992
F. Post-secretory events	Complexing with binding proteins	Vasilatos-Younken et al, 1991
G. Metabolic conversions	Fragments?	

Table 1.3. - Examples of Ectopic GH Producting Tumours

Tissue	Reference
Lung carcinoma	Sparanaga et al, 1971
	Beck & Burger, 1972
	Greenberg et al, 1972
Prostate gland	El Etreby & Mahrous, 1979
	Sibley et al, 1984
Stomach	Beck & Burger, 1972
Ovary	Kaganowicz et al, 1979
	Kallenberg et al, 1990
	Pilavdzic et al, 1993
Breast/Mammary gland	Kaganowicz et al, 1979
	El Etreby & Mahrous, 1979
Pancreas	Melmed et al, 1985
	Ezzat et al, 1993
Pharyngeal pituitary	Corenblum et al, 1980
	Warner et al, 1982

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CHAPTER 2. ECTOPIC EXPRESSION AND TRANSLATION OF THE GROWTH HORMONE GENE IN CHICKEN BRAIN

2.1. Introduction

Several of the hormones originally isolated from the anterior pituitary gland have now been reported to be present in the central nervous system (CNS) (Krieger, 1983). Due to the possible permeability of the blood brain barrier (BBB) to some peptides (Stern et al, 1975), and demonstration of retrograde flow from the pituitary gland (Bergland and Page, 1979), the origin of brain peptides remains uncertain. Growth hormone immunoreactivity (GH-ir) for instance, has been demonstrated in the cerebro-spinal fluid (CSF) of acromegalic humans (Linfoot et al, 1970; Hashimoto et al, 1986; Tamasawa et al, 1988) and high concentrations of GH are present in rat hypothalamic-hypophysial blood (Paradisi et al, 1993). Retrograde hypophysial portal flow may therefore be responsible for the presence of GH-ir in the brain. Immunocytochemical and radioimmunoassay studies have also demonstrated a wide distribution of GH-ir in the brain (Pacold et al, 1978; Kyle et al, 1981; Lechan et al, 1981; Hansen and Hansen, 1982; Hojvat et al, 1982a; Hojvat et al, 1986; Wright, 1986). The greatest concentration of immunoassayable GH appears to be present in the amygdala and hypothalamus (Hojvat et al, 1982b), and the molecule bears similarity to its pituitary counterpart, in terms of molecular size, antigenicity and biological effectiveness (Pacold et al, 1978). It is therefore possible that GH is synthesized in the brain, especially as immunoadsorption studies indicate the GH-like material in rat brain, is more closely related to the mid portion of the human (h) GH molecule than rat (r) GH (Lechan et al, 1983).

The origin of synthesis of brain GH is, however, uncertain. Cells originating from amygdaloid and hypothalamic nuclei, when grown in culture, release immunoreactive GH (Pacold et al, 1976; Pacold et al, 1977; Pacold et al, 1978). The possibility of endogenous synthesis of GH by the brain, is also supported by persistence of GH-ir in these nuclei after hypophysectomy (Hojvat et al, 1982b). Furthermore, in-situ hybridization with ^{32}P labelled GH cDNA probes, indicated that putative GH mRNA synthesis within the brain was widespread (Gossard et al, 1987),

although not in the hypothalamus, a principal site of ir-GH detection. These data nevertheless provide substantial evidence that brain GH represents a constituitive pool separate from that produced in the anterior pituitary.

The chicken provides an interesting model in which to study GH physiology. In this species circulating levels of GH in young birds are several orders of magnitude higher than in mammals (Scanes et al, 1983; Harvey et al, 1991). The hypersecretion of GH in young birds produces levels which would be considered pathological in man, similar to those seen in acromegaly (Harvey, 1987). Acromegaly often results from ectopic production of GH by tumours of the lung, pancreas and ovary (Greenberg et al, 1972; Ezzat et al, 1993; Pilavdzic et al, 1993). An ectopic site of GH synthesis may also explain the high GH levels in chickens and the persistence of low concentrations of GH in the plasma of hypophysectomized birds (Harvey et al, 1987; Lazarus & Scanes, 1988). The presence of high levels of GH in normal chickens may also facilitate detection of GH and its message in ectopic tissues. At present, the limited number of studies on brain GH, have focused on the rat and primate. In particular, no studies have addressed the possibility of GH production in the CNS of the chicken, in which pituitary GH chemistry, regulation and roles have been extensively examined Harvey et al, 1991; Scanes et al, 1993). The presence of neural GH in the chicken is therefore of considerable interest, especially as it has been hypothesized that GH and other peripheral hormones are phylogenetically evolved from neural peptides.

The possibility that the hypothalamus as well as extra-hypothalamic brain are sites of ectopic GH gene expression was therefore investigated in the present study using Northern blotting, reverse transcription and the polymerase chain reaction (RT-PCR). Translation of the GH gene and distribution of the gene product within these neural tissues was also examined.

2.2. Materials and Methods

Tissues

Pituitary glands and whole brains were rapidly dissected from 1 day old, 5 week, 10 week and adult (>20 week) White Leghorn chickens. The mediobasal

hypothalamus (MBH) was isolated from the rest of the brain tissue using the stereotactic co-ordinates of the avian hypothalamic nuclei (Kuenzel and van Tienhoven, 1988) as a guide. All tisssues were immediately frozen in liquid nitrogen and stored at -70°C prior to analysis.

To determine the influence of hypothyroidism on GH messenger ribonucleic acid (mRNA) levels, groups of chickens (12 males/group) also recieved 2ml thiourea to induce hypothyroidism (50ug/ml in 1.4ml of 0.1N NaOH made upto 400ml with 0.9% saline), or control injections (1.4ml 0.1N NaOH made upto 400ml with 0.9% saline), intraperitoneally (i.p.) for 3 weeks. After this time animals were sacrificed and RNA prepared for Northern analysis.

Radioimmunoassay (RIA)

The possibility that GH-ir may be present in the brain was first investigated by RIA. Tissues were homogenized (1g/10ml) in RIA buffer (0.04M NaPO4, 0.15M NaCl, 0.1% sodium azide, 0.01M EDTA, 0.5% ovalbumin; pH 7.0) and centrifuged at 2000 x g at 4°C for 30 min. Supernatants were boiled for 10 min and chicken (c) GH was measured using a double antibody RIA technique (Harvey and Scanes, 1977). Briefly, cGH was iodinated by the lactoperoxidase method (McIntyre et al, 1974). The reaction mixture contained 5ug hormone, 1 mCi [^{125}I]iodine (Amersham, Mississauga, ON, Canada), 10ug lactoperoxidase (0.35 units, Sigma Chemical Co, St.Louis, MO, USA) and 10ul 1.0 mM hydrogen peroxide. The reaction was terminated after 2 min by dilution with 0.5ml 0.05M sodium phosphate (pH 7) and labelled hormone had a specific activity of 102.5+/-5.3 (n=10) uCi/ug. Growth hormone was measured in a sample volume of 200ul and a final incubation volume of 400ul. Following a pre-incubation period with the primary antiserum (rabbit anti-cGH; final dilution 1:500) at 4°C for 24h, tracer was added (^{125}I-cGH; 10000 c.p.m.) and incubated for 24h at 4°C. Precipitating antiserum (donkey anti-rabbit Ig; Wellcome Laboratories) at a final dilution of 1:160 was then added and incubated for a further 24h at 4°C. The assay tubes were then centrifuged at 2000 x g at 4°C for 30 min, the supernatants discarded and the radioactivity in the precipitate was counted using an LKB gammamaster (Wallac OY, Turku, Finland). The IC_{50} was 3.67+/-0.37 ng/tube

and 10% inhibition 1.23+/-0.19 ng/tube. Intra-assay and inter-assay coefficient of variation were 6.4 and 17.75% respectively (Harvey and Scanes, 1977). Significant differences in ir-GH levels in different tissues was determined by students t-test.

Immunocytochemistry

The location of GH secreting neurons within the brain was assessed immunocytochemically. Sodium pentobarbitol anesthetized adult male chickens were perfused with approximately 300ml phosphate buffered saline (PBS: 137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.7H_2O$; 1.4mM KH_2PO_4) and then with an equal volume of Bouin's solution. Pituitary glands, whole brain and MBH were dissected and post fixed in Bouin's at 4°C overnight. Tissues were either cryoprotected by sequential saturation in 10%, 15%, 20% sucrose/PBS and frozen in isopentane, or dehydrated through a series of alcohols (50%, 70%, 80%, 90%, 100% respectively), cleared in hemo-de (Fisher Scientific, Ottawa, ON, Canada) and embedded in paraplast (Sigma). Sagittal sections (10μm) were cut and mounted onto gelatin (0.5%), chromium potassium sulphate (0.05%) coated slides, and immunocytochemical staining was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al, 1981) with commercial reagents (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Briefly, sections were incubated with a specific rabbit polyclonal antisera raised against cGH (Harvey and Scanes, 1977; Tai and Chadwick, 1977) diluted 1:5000- 1:8000 in PBS containing 1% normal rabbit serum (NRS) for 18h at room temperature, followed by two 10 min washes in PBS. Sections were then incubated in biotinylated goat anti-rabbit IgG (1:500) for 2h followed by two 10 min washes in PBS and treatment with ABC reagent for 1h. Staining was visualised using the chromagenic substrate diaminobenzidine tetrahydrochloride (DAB, 0.06%, Sigma). The specificity of staining was determined by preabsorbing the GH antiserum with recombinant cGH (Agen, Thousand Oaks, CA, USA; 1mg/ml) for 1h prior to incubation with sections. The presence of any non-specific staining was also determined by incubating the sections with pre-immune rabbbit serum rather than the primary antibody.

Immunoblot Analysis

The possible translation of the GH message in brain and pituitary gland was qualitatively assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blotting. Frozen tissue (extra-hypothalamic brain, MBH and pituitary glands) samples were homogenized (1g/10ml) in a solution containing 1% SDS, 1mM phenylmethylsulphonylfluoride (PMSF) and 10μg/ml aprotinin, using a Polytron homogenizer (Brinkman Instruments, IL, USA). Homogenates were centrifuged at 2000 x g for 5 min at 4°C and 30μg protein (determined by the Bradford method (1976)) was diluted 1:1 with loading buffer (0.06M Tris.HCl pH6.8; 10% glycerol; 2%SDS; 5% 2-β-mercaptoethanol; 0.001% bromophenol blue) and heated to 55°C for 15 min prior to loading. Proteins were then separated by electrophoresis in 15% SDS-polyacrylamide gels under reducing conditions, according to Laemmli (1970). After electrophoresis, the gels were equilibrated in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) and transferred electrophoretically (30V, 4h at 4°C) to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Non-specific binding sites on the membrane were blocked by incubating in 5% non fat dried milk, dissolved in Tris buffered saline (TBS: 25mM Tris.HCl, pH7.5; 0.5M NaCl) at room temperature for 1h. GH-ir was detected using a rabbit polyclonal GH antiserum (Harvey and Scanes, 1977; Tai and Chadwick, 1977), diluted 1:1000 in TBS/5% non fat dried milk, which was added to the membrane for 12h at room temperature. The membranes were then incubated with a horseradish peroxidase conjugated anti-rabbit IgG (Amersham) diluted 1:2000 in TBS/5% non fat dried milk. After washing, blots were developed with an enhanced chemiluminescence detection system (ICL Kit, Amersham) for 1 min and membranes were exposed to Kodak X-AR film (Kodak, Rochester, NY, USA) for between 30 sec to 2 min. The GH-ir on the blots was compared by laser densitometry.

Detection of glycosylated proteins

The possibility that the proteins detected might be glycosylated was subsequently determined. Tissue proteins were electrophoresed as above and the appropriate size band (approximately 26 KDa) was excised from the gel and

electroeluted with a Bio-Rad electroelution apparatus (Bio-Rad, Mississauga, ON, Canada) following manufacturers directions, for 4h at 60mA with elution buffer (25mM Tris; 192mM Glycine; 0.1% SDS). Protein was resuspended in fresh elution buffer and digested with glycosidase enzymes endoglycosidase F/PGNase F (0.1U; Sigma) or O-glycosidase (1mU; Sigma) according to manufacturers instructions. Samples were subjected to electrophoresis in 15% SDS-PAGE gels and transferred to Immobilon PVDF membranes (Millipore). GH-ir was visualized as previously described. Whole pituitary extracts were also digested with glycosidase enzymes and subjected to SDS-PAGE and subsequently stained with antisera raised against chicken luteinizing hormone (cLH) (Harvey et al, 1981), to determine the effectiveness of the digestion reactions and serve as positive controls. Carbohydrate moieties in glycoproteins were detected by incubating membranes with a concanavalin-A horseradish peroxidase conjugate (Con A-HRP: Sigma) and the ICL detection system (Amersham).

RNA preparation

GH gene expression in the chicken brain and pituitary gland was established by Northern blot analysis and the polymerase chain reaction (PCR). Total cellular RNA was extracted from the tissues by Polytron homogenization (Brinkman Instruments) in 5.5M guanidium thiocyanate containing, 0.25M sodium citrate, 0.5% (w/v) sodium lauryl sarcosine (pH 7.0), and 0.2% 2-β-mercaptoethanol. The supernatants were subjected to isopycnic ultracentrifugation (125,000 x g for 24h at 22°C) through a bed of caesium trifluoroacetic acid (Density 1.51±0.01g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), containing 0.1M EDTA (pH 7.0) (Fraser et al, 1991). RNA pellets were resuspended in diethylpyrocarbonate (DEPC) treated water and quantified spectrophotometrically at 260nm. Purity of RNA was assessed by the presence of 2 discrete bands of ribosomal RNA (18S and 28S) after electrophoresis on ethidium bromide stained 1% (w/v) agarose minigels (Maniatis et al, 1982).

Complementary deoxyribonucleic acid (cDNA) probe synthesis

A cDNA probe constructed from an 803 bp fragment of the cGH cDNA sequence inserted into the plasmid vector PUC119 was kindly donated by Dr Douglas Foster (Dept. Animal Science, University of Minnesota). This cDNA probe was then used to identify GH mRNA and cDNA on Northern and Southern blots respectively. The plasmid PUC119 was linearized by restriction enzyme digestion with *Eco R1* (5U/μg DNA; BRL, Burlington,ON, Canada) and the cDNA fragment was isolated from the vector DNA by electrophoretic seperation on 1.5% (w/v) agarose gels. The cDNA was then purified using Geneclean II (BioCan Scientific, Missisauga, ON, Canada) according to manufacturers instructions, and restriction endonuclease digested with *BstX1* and *Hae11* (5U/μg DNA; BRL) to produce a 613 bp fragment which did not contain the oligonucleotide primer sequences subsequently used in the PCR. The cDNA probe was then radiolabelled by the random priming method (BRL) in which the cDNA is incubated with short primers coding for a wide range of nucleotide sequences (Maniatis et al, 1982). Template cDNA was incubated with a nucleotide mix (dATP, dGTP and dTTP; 0.5mM each), ^{32}P labelled dCTP (Dupont, Mississauga, ON, Canada), and random hexamer primers, and synthesis of radiolabelled template was carried out by Klenow fragment (DNA polymerase 1; 3U). Radiolabelled template was purified from excess nucleotides and enzyme by Nuctrap push colums (Stratagene, La Jolla, CA, USA) and resuspended in double distilled water prior to incubation with membranes.

Northern Blot Analysis

To determine if GH mRNA was present in chicken neural tissues of normal chickens (1 day old, 5 week old, 10 week old or adult (>20 week old) or hypothyroid birds which have raised circulating GH levels, total tissue RNA (1-20μg) was electrophoresed through a 1.5% (w/v) agarose, 3.1% (v/v) formaldehyde gel, transferred to Hybond N nylon membrane (Amersham) by capillarity, and membranes were baked at 80°C for 2h under vacuum following a rinse in 6X SSC (1X SSC: 0.15M NaCl; 0.015M sodium citrate, pH 7.2). Membranes were prehybridized for 1-2h at 42°C in 30% formamide containing 6X SSC, 5X Denhardts (0.1% Ficoll, 0.1%

BSA, 0.2% SDS and 0.1% polyvinylpyrrolidone), 10% Dextran sulphate and salmon sperm DNA (50μg/ml) (Sigma). The membranes were then hybridized under the same conditions for 18h in the presence of the newly synthesized cDNA probe. After hybridization, the membranes were washed to remove non-specific radioactivity. Two 10 min washes in 2X SSC and 1% SDS at room temperature, followed by 15 min washes at 45°C in 1% SDS containing 1X, 0.5X and 0.1X SSC, respectively, were performed. Membranes were exposed to Kodak X-AR film (Kodak) between intensifying screens at -70°C and autoradiographed. The quantity of GH mRNA in normal and hypothyroid birds was determined by laser densitometry of the autoradiographs and corrected for loading error by comparison with the hybridization of a gamma-actin mouse cRNA probe (generously provided by Dr. R. Chibbar, University of Alberta) to the same blot. The levels of GH mRNA in different groups was compared by analysis of variance (ANOVA).

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

The presence of GH mRNA in chicken neural tissues was also assessed using RT-PCR. Total tissue RNA (1μg) was reverse transcribed by superscript (100U; BRL) in the presence of 50pmol 3'-oligomer cGH antisense primer based on the known sequence of the cGH (Lamb et al, 1988) (GCCTCAGATGGTGCAGTTGCTCTCTCCGAA), or 100pmol oligodeoxythymine primer (Boehringer Mannheim, Dorval, Quebec, Canada), excess deoxynucleotides (10mM each of dATP, dCTP, dGTP, dTTP; Boehringer Mannheim) and 5X H-RT buffer (BRL). The reactions were diluted with double distilled water (50:1 v/v) and an aliquot of each (0.5% of total volume) was added to a PCR mixture (Kawasaki, 1990) containing both 5'-oligomer sense (CGTTCAAGCAACACCTGAGCAACTCTCCCG) and 3'-oligomer antisense (GCCTCAGATGGTGCAGTTGCTCTCTCCGAA) primers (Nucleotide Synthesis Laboratory, University of Alberta, Edmonton, Alberta, Canada) for cGH (15pmol each), deoxynucleotides (200μmol of each), 1X PCR buffer (80mM KCl; 16mM Tris-Cl, pH 8.4; 1.5mM MgCl2; and 0.1% Triton X-100), and *Thermus aquaticus* (*Taq*) DNA polymerase (5U, Promega, Madison, WI, USA). The mixture was overlayed with mineral oil (v/v) to prevent evaporation, and denatured at 94°C

for 2 min prior to 30 cycles of denaturing (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 2 min), with a final extension (72°C for 10 min) in a thermal cycler (MJ Research, Watertown, MA, USA). Reaction products were analysed by electrophoresis in ethidium bromide stained 1.5% (w/v) agarose gels and compared with DNA molecular weight markers (ϕX174 RF DNA/*Hae III*; Sigma).

Identification of cGH in neural tissues

Fragments generated by RT-PCR were restriction endonuclease digested with *Bam H1* or *Rsa 1* (5U/μg DNA; BRL) for 2h at 37°C to determine if the fragments contained the same restriction endonuclease sites as pituitary GH cDNA. Products were identified by electrophoresis of reactions in ethidium bromide stained 1.5% (w/v) agarose gels. PCR fragments were also analysed by 1.5% (w/v) agarose gel electrophoresis and Southern transfer to Hybond N nylon membranes (Amersham) for 18h with 20X SSC. Membranes were baked at 80°C for 2h under vacuum and prehybridised for 3h at 42°C in 50% formamide containing 5X SSC, 5X Denhardts (0.1% Ficoll, 0.1% BSA, 0.2% SDS, 0.1% PVP), 1% SDS and 50μg/ml salmon sperm DNA (Sigma). Hybridization was performed under the same conditions for 18h in the presence of 10% dextran sulphate and newly synthesized cDNA probe. After hybridization membranes were washed for 15 min at 60°C with 2X, 1X, 0.5X and 0.1X SSC respectively, containing 0.1% SDS, and membranes were exposed to Kodak X-AR film (Kodak) between intesifying screens for 1-24h at -70°C.

PCR sequencing

To confirm the possibility that the PCR products generated from brain tissues were related to the GH mRNA sequence, sequence analysis of the cDNAs was performed. PCR reaction products were electrophoresed in 1.5% (w/v) agarose ethidium bromide stained minigels (Maniatis et al, 1982). The appropriate size ethidium staining DNA band (774 bp) was excised from the gel and purified from excess nucleotides and agarose using Geneclean II (BioCan Scientific), according to manufacturers instructions, the fragment was then resuspended in double distilled water prior to sequencing. PCR fragments were cycle sequenced (both strands) by a

modified cycle dideoxy chain termination method (Sanger et al, 1977). Briefly, 2pmol template DNA was added to a mixture of 5X sequencing buffer (250mM Tris.HCl pH9; 10mM $MgCl_2$: Promega), 5'- oligomer sense (CGTTCAAGCAACACCTGAGCAACTCTCCCG) or 3'-oligomer antisense (GCCTCAGATGGTGCAGTTGCTCTCTCCGAA) primer (4.5pmol), sequencing grade *Taq* DNA polymerase (Promega) and one of the 4 silver sequence deoxy/dideoxy nucleotides (d/ddATP, d/ddCTP, d/ddGTP, or d/ddTTP). The mixture was overlayed with mineral oil (v/v) and heat denatured at 95°C for 2 min before 55 cycles of 95°C for 30 sec (denaturing step) and 70°C for 30 sec (annealing /extension step) in a genetic thermal cycler (MJ Research). The reaction was terminated by addition of DNA sequencing stop solution (10mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanol). cDNA from each tissue was sequenced 5-6 times to increase fidelity of results. Sequenced products were heated at 70°C for 2 min prior to loading on 6% (v/v) acrylamide/bis-acrylamide gels (19:1), containing 1X TBE (0.089M Tris; 0.089M Boric acid; 20mM EDTA pH8) and electrophoresed at 1800V for 1.5 to 2.5h. After electrophoresis the gel plates were separated and the gel fixed in 10% glacial acetic acid, stained with silver nitrate (1g/L) solution, containing 0.05% formaldehyde, and developed with chilled (10 - 12°C) Na_2CO_3 solution (30g/L), containing sodium thiosulphate (2mg/L) and formaldehyde (0.05%). The developing reaction was terminated by addition of 10% glacial acetic acid (v/v), and gels were air dried, prior to exposure to EDF film (Kodak) for 10 to 15 seconds. The deduced nucleotide sequences were compared with the published sequence for cGH cDNA (Lamb et al, 1988).

2.3. Results

Radioimmunoassay

Serial dilutions of the chicken hypothalamus and pituitary gland extracts displaced the binding of ^{125}I-labelled GH to the GH antisera in a manner parallel to that of the standard (fig 2.1). The GH-ir in the MBH extract (9.52ng/mg protein) was greater ($P<0.01$) than that in extra-hypothalamic brain (1.21ng/mg protein), but only

25% ($P<0.01$) of that in pituitary extract (38.09ng/mg). Other tissue extracts, including muscle, duodenum and liver appeared to have no significant GH-ir (< 0.1ng/mg protein).

Immunocytochemistry

The distribution of GH-ir in chicken pituitary gland, whole brain and MBH was investigated using a polyclonal antibody raised against cGH. As expected, this antibody identified GH positive cells distributed evenly throughout the caudal lobe of the anterior pituitary gland. A few cells in the cephalic lobe also stained positive for GH-ir (fig 2.2). A complete loss of staining was observed after preabsorption of the primary antibody with rcGH (1mg/ml) (data not shown). In contrast, no peroxidase reaction product was observed after incubation of whole brain or MBH sections with the cGH antisera (data not shown).

Western analysis

The presence of a GH like protein in neural tissues of the chicken was assessed by immunoblotting, using a polyclonal antibody raised against cGH. At least 4 immunoreactive bands of 16, 22, 26, and 44 kDa were detected in the pituitary gland (Fig 2.3). In contrast, a major immunoreactive band of approximately 26 kDa was detected in hypothalamic and extra-hypothalamic brain tissue, which co-migrated with the major fraction of immunoreactivity in the chicken pituitary gland. A faint 44 kDa GH-ir band was also detected in hypothalamus and extra-hypothalamic brain. The intensity of the 26 kDa protein in the pituitary gland (3.21 optical density (O.D.) units) was consistently more than that in hypothalamic (1.35 O.D. units) or extra-hypothalamic brain (1.06 O.D. units). Immunoadsorption of the primary antibody with rcGH (1mg/ml) abolished the banding pattern (fig 2.3b) and no non-specific staining was observed when membranes were incubated with pre-immune serum (fig 2.3c).

After electroelution to partially purify the 26 kDa immunoreactive bands, they were digested with glycosidase enzymes. A single GH-ir band was detected in pituitary gland and brain tissues after size selection. These proteins were however, resistant to glycosidase digestion (Fig 2.4a and 2.5a). In contrast LH was cleaved

under the same conditions by both enzymes, as expected. As fig 2.4b indicates, a reduction in the size of the LH immunoreactive band from 32 kDa to 28 kDa was observed after digestion with O-glycosidase. In addition, a complete loss of LH immunoreactivity was seen following endoglycosidase F treatment (Fig 2.5b). An absence of glucose or high mannose glycolytic components in these proteins was also demonstrated by analysis of tissue extracts using SDS-PAGE and staining with Con-A-HRP. This indicates the absence of glucose or high mannose carbohydrate chains in the 26 kDa GH-ir material in pituitary, extra-hypothalamic brain and hypothalamus (Fig 2.6). Slight staining of high molecular weight bands was, however, observed in whole tissue extracts which had not been previously size selected for the GH-ir band.

Northern Analysis

Hybridization of total RNA extracted from chicken pituitary glands with the cGH cDNA probe is shown in figure 2.7a. The cDNA probe hybridized to a single RNA moiety of approximately 800bp in pituitary gland (lanes 1 and 2); however no hybridization signal was apparent with hypothalamus (lane 3) or extra-hypothalamic brain (lane 4) RNA at any of the tested ages (1 day old, 5 week, 10 week, adult (>20 week)). A hybridization signal was detected in pituitary gland using 2-5μg RNA, however, no signal was apparent even after using 20μg of brain or hypothalamic RNA, and overexposing the blot for up to 3 weeks did not reveal any hybridization signal . Similarly, treatment of birds with thiourea to induce hypothyroidism, increased mRNA levels in pituitary gland (3.04 compared to 1.16 O.D. units ; $P<0.001$), but no GH message was detected in hypothalamic or extra-hypothalamic brain RNA from thiourea treated birds (figure 2.7b).

PCR

In the presence of 3' and 5'- oligonucleotide primers for the cGH sequence (fig 2.8), a single cDNA moiety of 689 bp was synthesized from chicken hypothalami and extra-hypothalamic brain regions by PCR. These PCR products comigrated with the cDNA produced from the pituitary gland, when visualised on ethidium bromide stained 1.5% agarose gels (fig 2.9a). Digestion of hypothalamic and brain cDNA with

the restriction endonuclease *Bam H1* produced two bands of approximately 465 and 224 bp, identical to products of *Bam H1* digestion of pituitary cDNA (fig 2.9b). *RSa 1* digestion of these cDNA products also produced fragments of expected size (237 and 452 bp) (data not shown). In contrast, no detectable GH like cDNA fragments were generated when muscle or liver cDNA was amplified. cDNA moieties were also not generated when RNA or reverse transcription was omitted from the reverse transcription reaction.

Southern Blot

Hybridization of the cGH cDNA probe to the cDNA generated from extra-hypothalamic brain and MBH by PCR was observed within 2h of exposure. The probe hybridized to a single cDNA moiety in these tissues (approximately 770bp), and also to a similar sized cDNA generated from the pituitary gland. As figure 2.10 shows, this hybridization was specific, as no signal was detected in PCR products produced from muscle tissue. PCR products were similarly not generated if RNA or enzyme was omitted from the reaction during the initial reverse transcription. To exclude the possibility that the probe was hybridising with the primers used to generate the cDNA in the PCR, the probe was digested with *BstX1* and *Hae11* to remove these sequences prior to hybridization.

PCR sequencing

Sequence analysis of cDNAs produced by PCR from both extra-hypothalamic brain and isolated MBH was performed using a modified cycle dideoxy chain termination method. PCR products from both MBH and extra-hypothalamic brain demonstrated complete homology with the published cGH cDNA sequence after sequencing 594 and 550 bp of their respective lengths (Fig 2.11). In addition the same sequence homology existed between PCR reactions from pituitary gland and those of brain and hypothalamus. This identical sequence was observed on sequencing both sense and anti-sense strands of the cDNA on acrylamide gels and staining the banding pattern with silver nitrate (Fig 2.12).

2.4. Discussion

The results of this study indicate the ectopic expression and translation of the GH gene in the chicken brain. The presence of a GH-like molecule, similar if not identical to its pituitary counterpart has been reported previously (Pacold et al, 1978; Hojvat et al, 1982a; Hojvat et al, 1986). This is the first study, however, to demonstrate that the expression of the GH gene in neural tissues may be responsible for the presence of GH-ir in the same brain regions. This colocalization of GH mRNA and protein in the MBH and extra-hypothalamic brain tissues suggest roles for endogenous GH in the chicken brain.

The identification of a 26 kDa GH-ir protein in both hypothalamic and extra-hypothalamic brain tissues by SDS-PAGE and immunoblotting with a polyclonal antibody raised against pituitary cGH, along with the finding of parallel displacement curves in RIA, suggests that the brain and pituitary GH are immunologically similar. However, although the same antibody recognised GH-ir in paraffin and frozen sections of the chicken pituitary gland, no GH-ir could be detected on sections of MBH or whole brain using immunocytochemistry. This inconsistency may be explained by the experimental treatments of the proteins under analysis. In the immunoblotting procedure, treatment with SDS and mercaptoethanol results in disruption of the disulphide bonds, therefore altering structural conformation of the protein. This may reveal epitopes which would not be apparent in the unreduced state of the proteins in immunocytochemistry. Similarly, fixation of the neural tissues with Bouin's solution may have destroyed some of the epitopes of the GH-like molecules.

Brain GH in rats has been shown to more closely resemble the mid-portion of the hGH molecule than native rGH (Lechan et al, 1981). This suggests that although closely related, the GH-ir in brain may not be identical to pituitary GH. Therefore, the use of a more comprehensive panel of antibodies may be required to determine ectopic immunoreactivity distribution. The use of a rat or human GH polyclonal antisera which recognises different epitopes may have been a better choice for this investigation, and subsequent studies should employ such antisera.
Radioimmunoassay also indicated the MBH contained only one quarter of the GH-ir of the pituitary gland and extra-hypothalamic brain even less. The MBH is rich in axonal

projections, but does not contain the cell bodies presumably responsible for production of GH-like proteins. Therefore, low levels of GH-ir may explain the lack of immuno-staining in the MBH. However, no staining was observed in sections of whole brain where cell bodies would be present. This suggests that brain GH-ir is either not in sufficient concentration, or is not recognised by the antibody used in this study. Inadequate fixation of the sections may be responsible for the lack of staining, but this seems unlikely as other tissues were adequately fixed using the same procedure.

In the chicken a heterogenous pattern of GH has been demonstrated in pituitary extracts and purified hormone preparations (Houston and Goddard, 1988; Aramburo et al, 1989; Aramburo et al, 1990). Earlier reports on the molecular size of monomeric cGH have ranged from 22 to 27 kDa (Harvey and Scanes, 1977; Lai et al, 1984; Souza et al, 1984; Houston and Goddard, 1988), depending on the experimental techniques employed for analysis. In addition a glycosylated form of cGH has been described, which is in the size range of 25 to 29 kDa when analyzed by SDS-Page and immunoblotting procedures (Berghman et al, 1987; Aramburo et al, 1991). In the present study GH-ir bands of 16, 22, 26 and 44 kDa were identified in the pituitary gland. In contrast, the 26 kDa band represented most of the GH-ir in hypothalamic and extra-hypothalamic brain tissue, and a minor GH-ir band of 44 kDa was also present. The degree of variation in the sizes of the monomeric and glycosylated forms of GH found in different studies, along with the considerable overlap between the sizes of these two species, led us to investigate whether the GH-ir in neural tissue included a portion which was glycosylated. Digestion of the 26 kDa GH-ir species with O-glycosidase or endoglycosidase F/PGNase F, indicated that this GH-ir did not contain high mannose or complex carbohydrate moieties joined by the glycosidic bond of the N, N'-diacetylchitobiose core structure, O- or N-linked to asparagine . Moreover, whole tissue homogenates analyzed after incubation with the lectin Con-A, did not appear to contain any glucose/high mannose carbohydrate chains in proteins with a molecular weight corresponding to any of the GH-ir bands in pituitary or neural tissue. It is, however, possible that this protein is glycosylated by other carbohydrate linkages or groups. The 26 kDa GH-ir band observed in these experiments under

reducing conditions, may therefore be a result of cleavage of intra-chain disulphide bonds in the monomer 22 kDa protein. This would inhibit migration of the proteins in acrylamide gels, and indicate an increased apparent molecular weight, rather than represent the presence of a genuine variant (Houston and Goddard, 1988). The presence of the 22 kDa GH-ir band in pituitary extracts may represent an incompletely reduced portion of monomeric GH, which retains its intra-chain disulphide bonds, thereby conferring a more stable conformation. The occurence of the immunoreactive 22 kDa GH moieity soley in the pituitary gland, may reflect the greater abundance of GH in this tissue and/or a tissue-specific difference in GH translation and processing. The 16 kDa GH-ir species present in pituitary extract most likely represents a proteolytic fragment of the 22 kDa GH (Aramburo et al, 1990), although an alternate splicing pattern of mRNA in human pituitary tumour tissue also predicts a protein of 17 kDa (Lecomte et al, 1987). A small fraction of the pituitary GH-ir appears as a 44 kDa species, probably representing a dimeric form of GH resistant to reduction by mercaptoethanol. Faint GH-ir bands of 44 kDa are also present in hypothalamic and extra-hypothalamic tissue. These incompletely reduced forms may be a covalent dimer linked by bonds other than inter-chain disulphide bonds, as has been described for human GH (Lewis et al, 1977).

These results clearly demonstrate the presence of GH-ir in the hypothalamus, extra-hypothalamic brain and pituitary gland of the chicken. The present findings are in agreement with those of Pacold et al (1978), who detected GH-ir in perikarya and fibres in the hypothalamus and pre-optic nucleus of rat brain. More recently GH-ir has been described in the brains of lower vertebrates (Hansen and Hansen, 1982; Wright, 1986), which may be of evolutionary significance, as no GH-ir could be detected in the anterior pituitary gland of the sea lamprey (the living representative of the earliest vertebrate group, the Agnatha). The presence of GH-ir in neural tissues of the chicken may indicate that these are ancestral sites of GH production. In addition, the observation that GH-ir in the rat brain is not affected by hypophysectomy (Hojvat et al, 1982b) suggests that these peptides originate in the CNS rather than the pituitary gland.

The possibility that the GH-ir in the brain reflects an ectopic site of GH production is supported by the demonstration of GH mRNA in the chicken hypothalamus by PCR. The cDNA of the GH gene expressed in the hypothalamus has endonuclease restriction sites and nucleotide sequence identical to that in the pituitary gland, as confirmed by Southern blotting. Thus, although a family of GH or GH-like genes are expressed in primates, rats and fish (Parks, 1989; Male et al, 1992; Ber et al, 1993; Golos et al, 1993) these results demonstrate for the first time that the GH gene ectopically transcribed in neural tissue, is identical to the pituitary GH gene.

The occurence of GH mRNA in the chicken brain is likely to reflect constituitive expression. Using oligonucleotide primers specific for the cGH mRNA sequence, a cDNA of expected size was amplified from both hypothalamus and extra-hypothalamic brain, indicating GH gene expression in these tissues. Recently the phenomenon of "illegitimate" (Chelley et al, 1988) or "ectopic" transcription (Sarkar and Sommer, 1989), which amplifies rare transcripts in tissues previously thought not to express the gene sequences has been demonstrated. No physiological role has been attributed to this illegitimate transcription, but Chelley et al (1989) have suggested that it may be due to a permanent basal level of transcription of 'any gene in any cell'. However, demonstration of this phenomenon necessitates a technique called booster PCR (Ruano et al, 1989). In this process an initial PCR of 30-35 cycles is followed by a second round of 30-35 cycles of PCR. As GH cDNA was visible after only a single PCR of 30 cycles in the present study, this appears to represent a substantial level of GH mRNA signal in the brain.

Although a previous study has demonstrated by *in situ* hybridization, the presence of GH mRNA in the rat brain, we are the first to demonstrate the coincident localization of the GH message and its translated product in brain tissue. The abundance of the GH message in the chicken brain would, however, appear to be low, since it could not be detected by Northern blotting. Steady-state levels of GH mRNA in the chicken hypothalamus could not be detected in birds in which circulating GH levels and pituitary GH mRNA were elevated during ontogeny or enhanced by hypothyroidism. In addition, the relative amounts of GH-ir and GH mRNA detected in the pituitary gland were not reflected by a corresponding relationship in the chicken

hypothalamus. It is therefore probable that the turnover of GH mRNA in the hypothalamus differs from that in the pituitary gland. Alternatively, the constituitive transcription and regulated expression of the GH gene may occur at levels in the chicken brain that are much lower than those in the pituitary gland, even though GH mRNA was readily detected by PCR. These results also suggest that GH gene expression may not be tissue-specific.

The expression of the cGH gene in the brain raises a number of questions about the mechanism of gene activation of this "so-called" pituitary specific gene in an extra-pituitary site. In humans and rats, this pituitary-specific expression has been attributed to the gene promoter regions, which contain two cis-elements for binding of the pituitary-specific transcription factor Pit-1/GHF-1 (Bodner et al, 1988; Ingraham et al, 1988). Pit-1/GHF-1 is generally thought to be restricted to pituitary somatotrophs, lactotrophs and thyrotrophs (Simmons et al, 1990). Indeed Pit-1 mRNA could not be identified in the brain of rats (Emanuele et al, 1992; Delhase et al, 1993), suggesting that it does not play a role in the regulation of brain GH gene transcription. It is known that genomic alterations may activate genes independently of their tissue specific regulatory mechanisms. Inappropriate gene activation might result from the loss or acquisition of regulatory cis-elements through DNA rearrangements, insertions or deletion. Differences in the sequence of the promoter region may therefore be responsible for expression of the GH gene in extra-pituitary tissues of the chicken. The sequence of the GH promoter is highly conserved in mammalian genes (Vize et al, 1987). In contrast, the cGH gene promoter region has no overall sequence homology with those in other species (Tanaka et al, 1992). No apparent concensus sequences for hormone response elements have been identified in the cGH promoter region. However, a sequence which is highly homologous to the antisense strand sequence in the rat GH gene promoter for Pit-1 binding has been identified. These differences may indicate a differential pattern of GH gene regulation in the chicken.

In summary these results are the first to demonstrate the expression of GH mRNA in neural tissues of the chicken. The detection of GH-ir in these tissues suggests a local synthesis of GH in the brain, where it may act as an autocrine or paracrine factor contributing to the ontogeny, differentiation and function of neural

tissues in avian species. In addition, the ectopic production of GH may explain the persistence of GH in the plasma of hypophysectomized birds and the hypersecretion of GH in immature fowl.

2.5. Figures

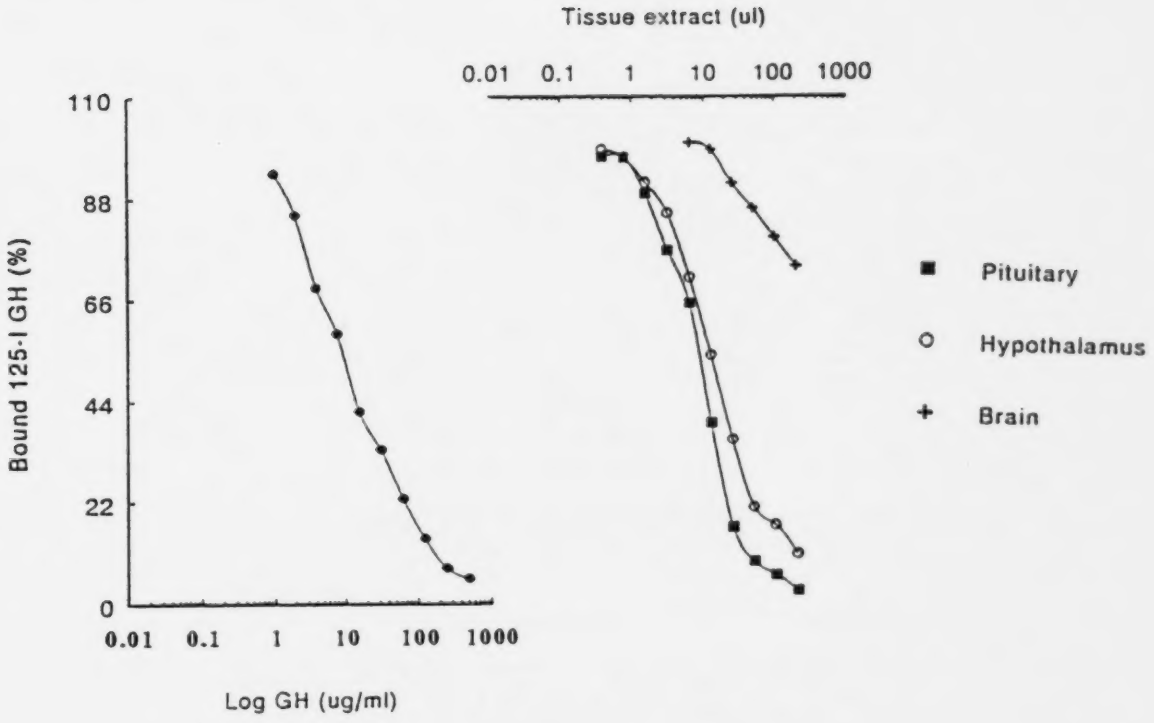

Figure 2.1 - Cross reaction of crude saline homogenates of chicken pituitary gland, hypothalamus and extra-hypothalamic brain with cGH antisera. Dose response curves for neural tissue extracts showed parallelism with the standard curve. The slopes of the lines lay within the 95% confidence limits for the GH standard (-0.57 to -0.64).

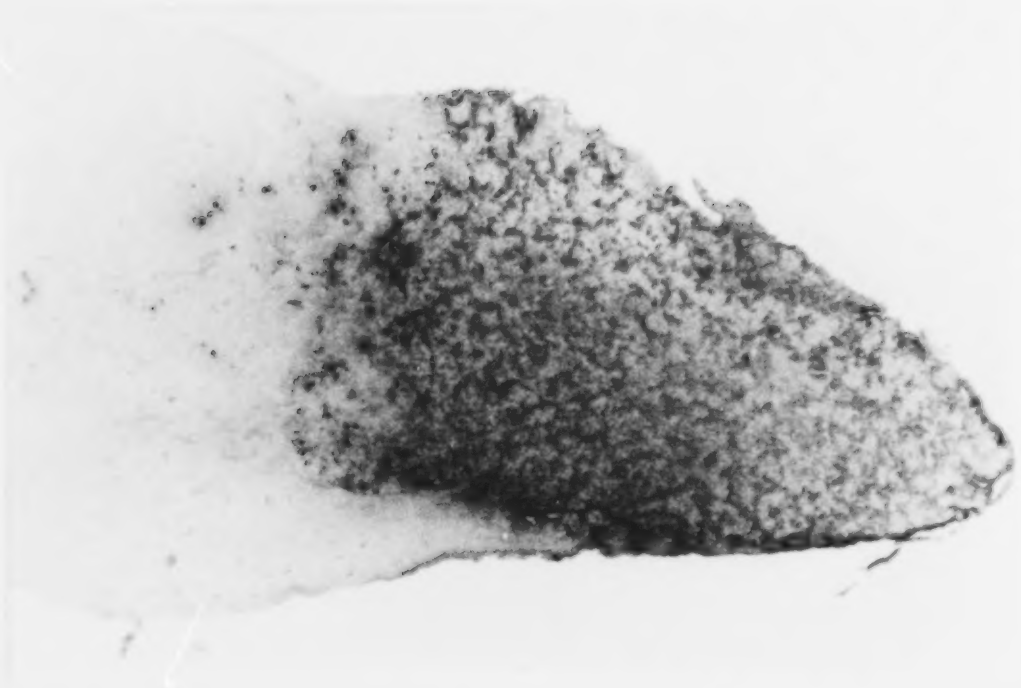

Figure 2.2 - Sagittal section through the chicken pituitary gland demonstrating the location of immunoperoxidase staining after incubation with a rabbit polyclonal antibody raised against cGH.

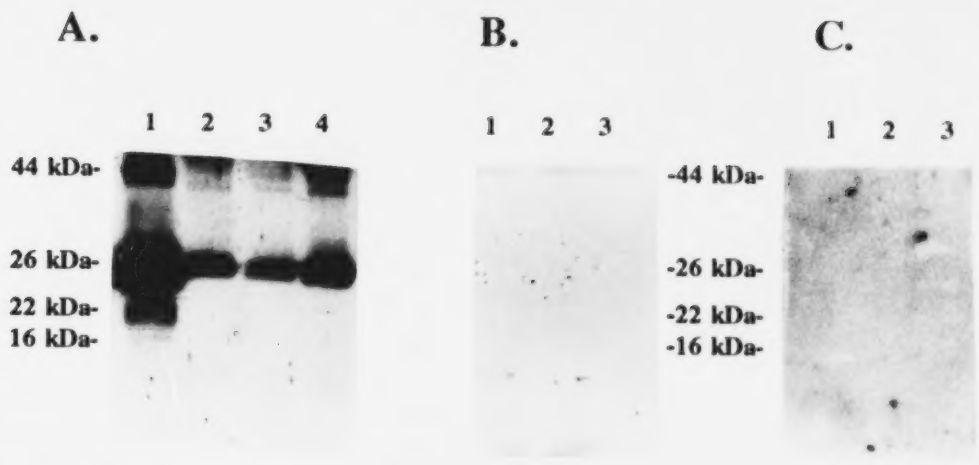

Figure 2.3 - Immunoblot detection of GH in neural tissues. Immunoblotting procedures were performed as described using a polyclonal antibody raised against cGH at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1;2000. (A) Lane 1, pituitary homogenate; lanes 2 and 3, extra-hypothalamic brain sample; and lane 4, medio-basal hypothalamic extract. Approximately 30ug protein as determined by the method of Bradford (1976) was loaded in each lane. (B) Preabsorption of α-cGH with rcGH (1mg/ml). (C) Incubation with Normal rabbit serum.

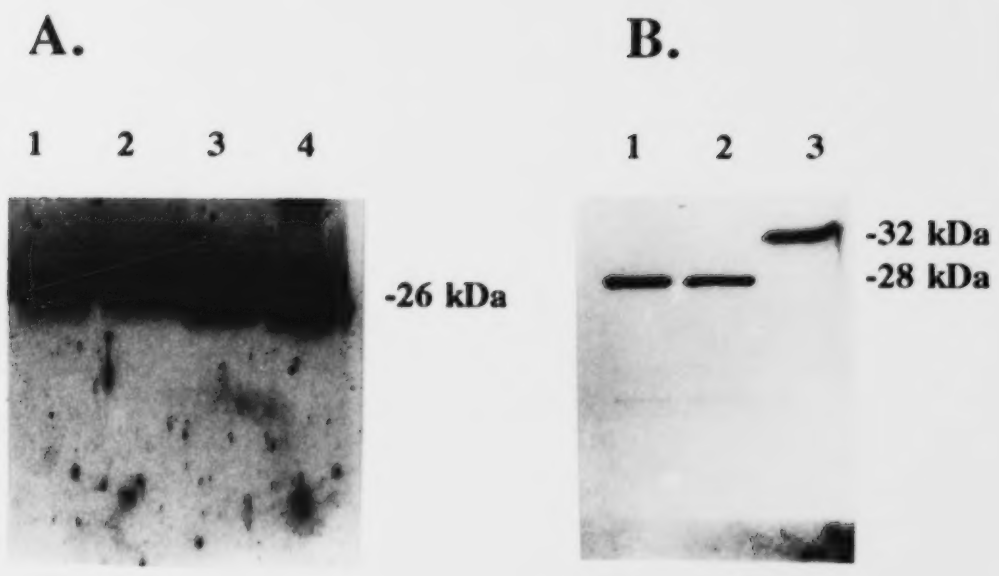

Figure 2.4 - Analytical SDS-PAGE on 15% gels, under reducing conditions of the products of O-glycosidase digestion. (A) Stained with α-cGH (1:1000). Lanes: (1) Hypothalamus; (2 and 3) Brain; (4) Pituitary. (B) Stained with α-cLH (1:1000). Lanes (1 and 2) Digested pituitary extract; (3) Undigested pituitary extract.

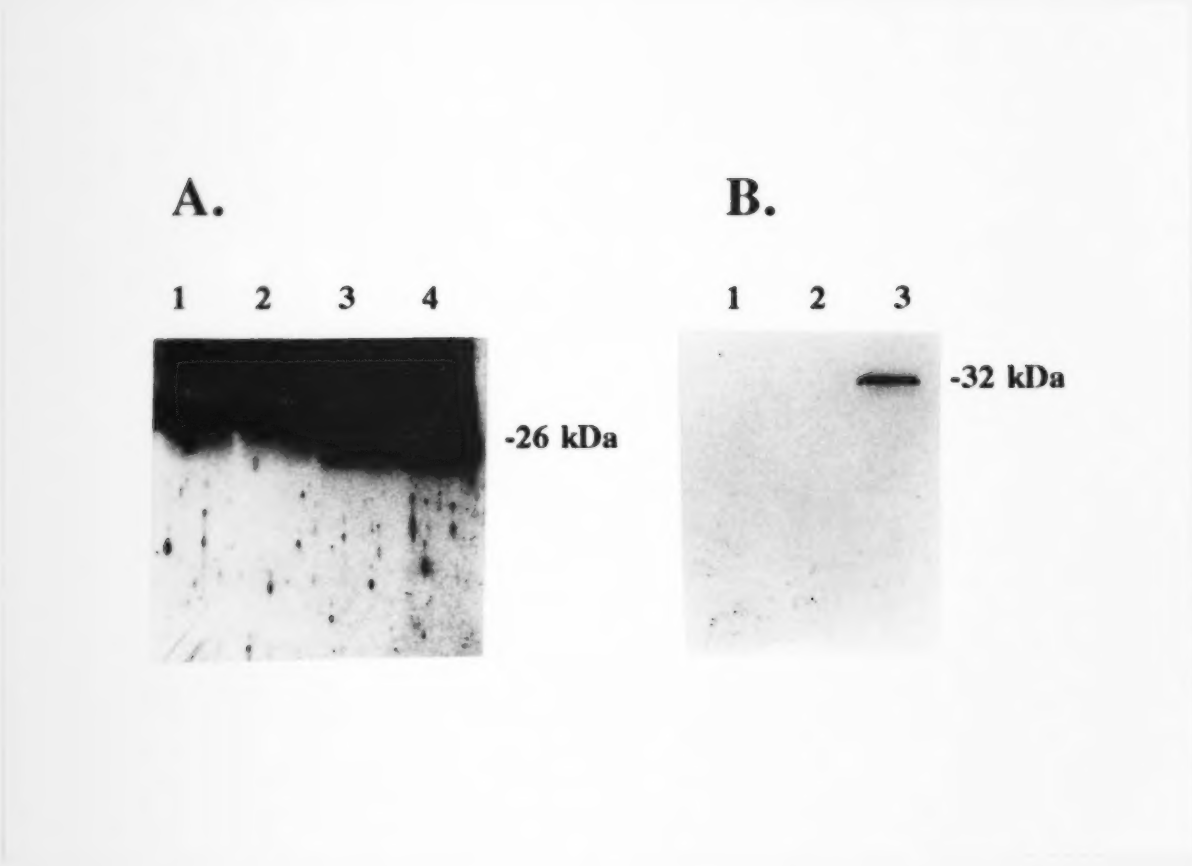

Figure 2.5 - Analytical SDS-PAGE of on 15% gels, under reducing conditions of the products of N-glycosidase F/PGNase F digestion. (A) Stained with α-cGH (1:1000). Lanes: (1) Hypothalamus ; (2 and 3) Brain; (4) Pituitary. (B) Stained with α-cLH (1:1000). Lanes (1 and 2) Digested pituitary extract; (3) Undigested pituitary extract.

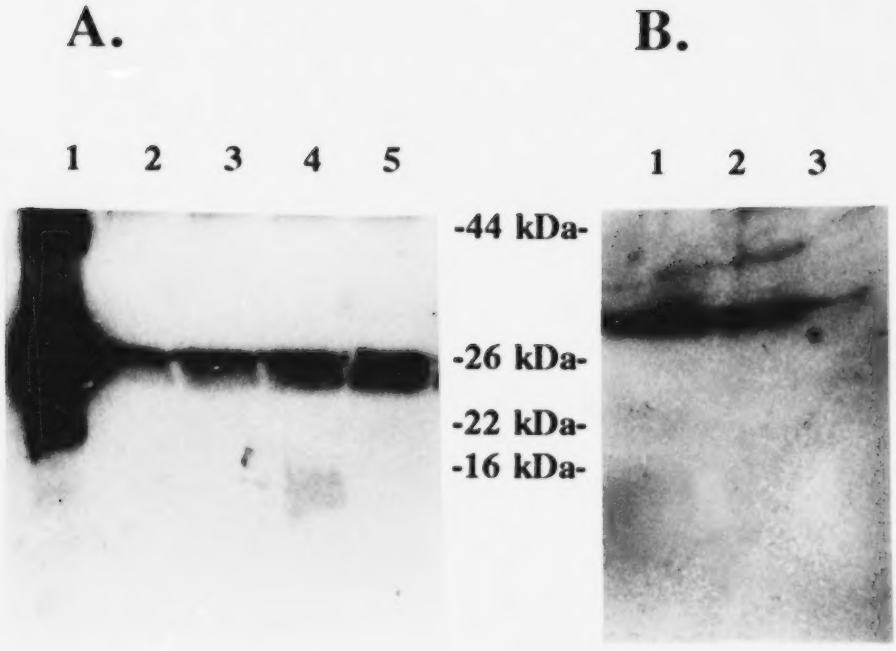

Figure 2.6 - (A) Analysis of cGH on SDS-PAGE immunoblots. Stained with α-GH (1:1000). Lanes (1) Pituitary extract; (2 and 3) Brain; (4 and 5) Hypothalamus. (B) Analysis of glycosylated proteins on lectin blots stained with a Con A horseradish peroxidase conjugate (1:1000). Lanes (1) Pituitary; (2) Brain; (3) Hypothalamus.

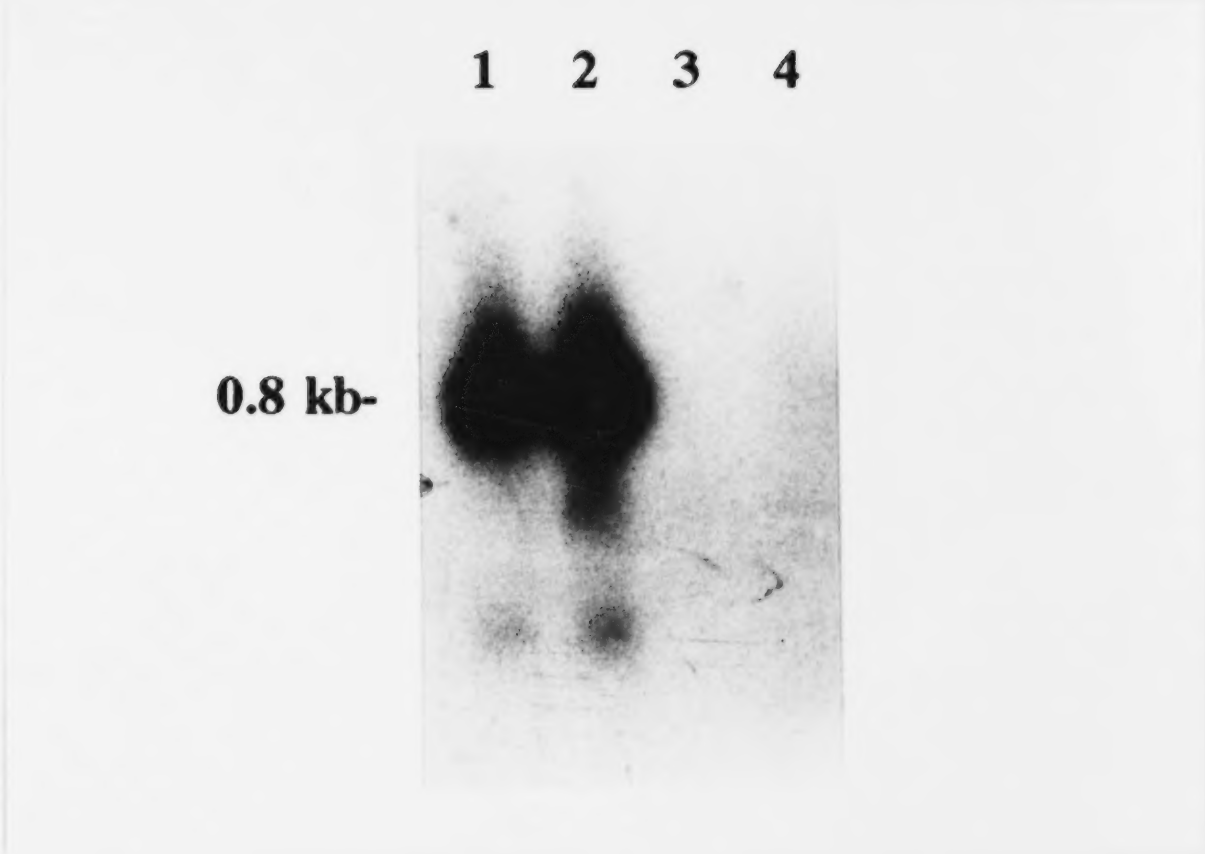

Figure 2.7 - A representative Northern blot representing the cross-hybridization of a complementary DNA (cDNA) probe for chicken GH with total cellular RNA extracted from chicken pituitary gland (lanes 1 and 2), medio-basal hypothalamus (lane 3) and extra-hypothalamic brain (lane 4).

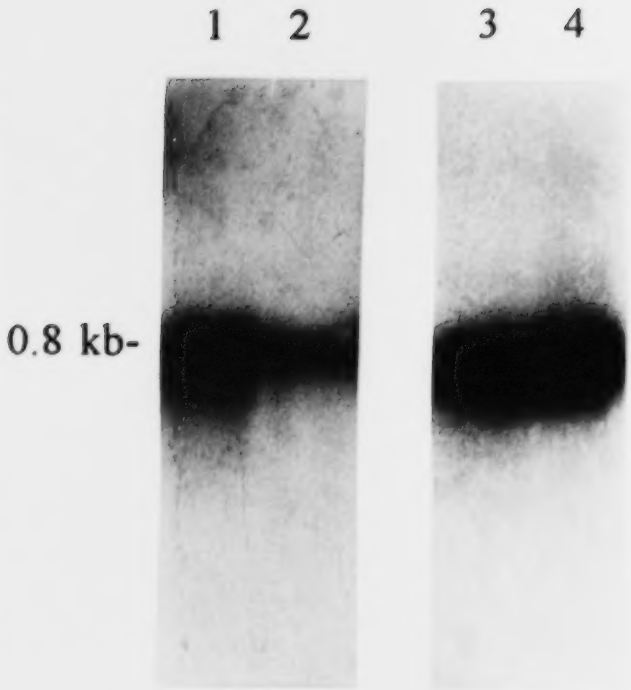

Figure 2.7a - Hybridization of a complementary DNA probe for chicken GH with total cellular RNA (5ug) extracted from pituitary gland of control (lanes 1 and 2) and hypothyroid (lane 3 and 4) birds.

A.

Primers

cre1: 5' gcctcagatggtgcagttgctctctccgaa 3'

cre2: 5' cgttcaagcaacacctgagcaactctcccg 3'

B.

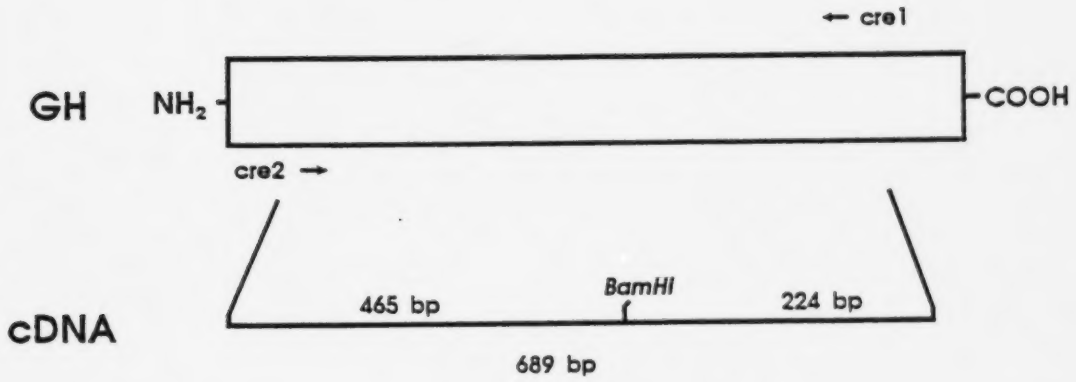

Figure 2.8 - (A) Oligonucleotide primers (CRE 1 and CRE 2) used in the polymerase chain reaction (PCR) to amplify the coding region of the GH gene. (B) Sites of primer annealing and restriction endonuclease digestion site on the GH cDNA.

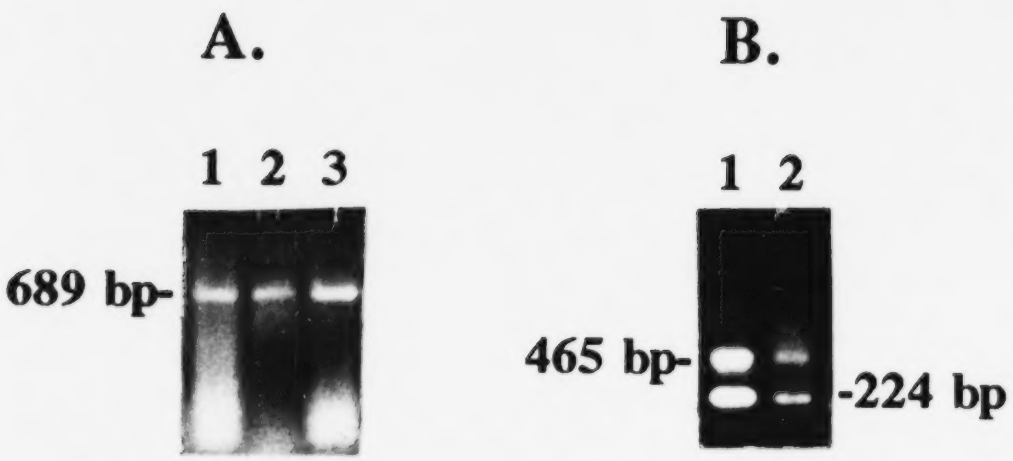

Figure 2.9 - Ethidium bromide stained 1.5% agarose gel through which cDNA was electrophoresed. The size of the fragments was determined by comparison with ΦX174RF DNA/Hae III size markers. (A) The cDNA was reverse transcribed from total RNA extracted from chicken hypothalamus (lane 1), extra-hypothalamic brain (lane 2), and pituitary gland (lane 3) and amplified in the presence of oligonucleotide primers CRE1 (exon 1) and CRE2 (exon 5). (B) Bam H1 digests of pituitary (lane 1) and hypothalamic (lane 2) cDNA are also shown.

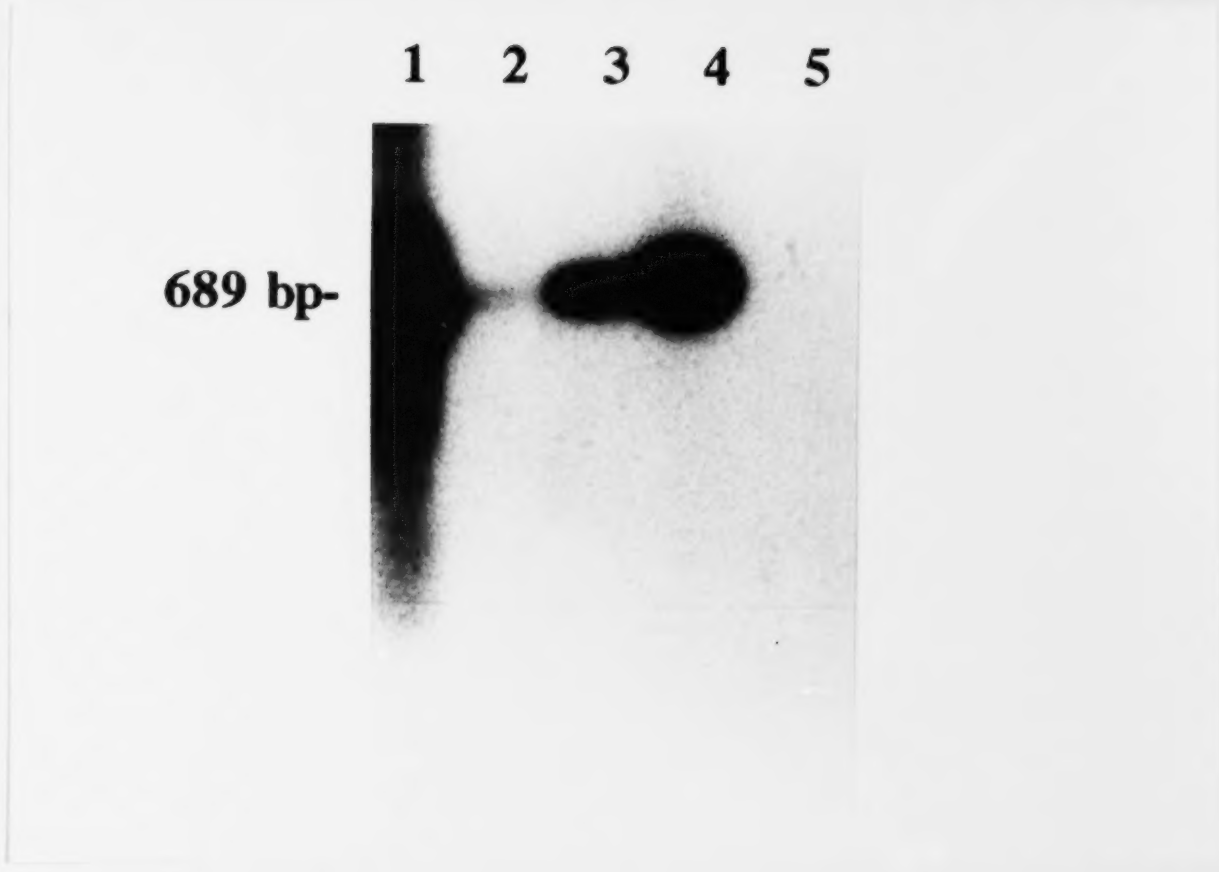

Figure 2.10 - Southern analysis. RT-PCR products amplified from chicken pituitary (lane 1), muscle (lane 2), brain (lane 3) and hypothalamic (lane 4) RNA were southern transfered and analysed using a radiolabelled cGH cDNA as an internal probe. A negative control which represents amplification of first strand cDNA synthesis lacking RNA is also included (lane 5).

100 (815)
AGGCTCGTGGTTTTCTCCTCTCCTCATCGCTGTGGTCACGCTGGGACTGC

150 (865)
CGCAGGAAGCTGCTGCCACCTTCCCTGCCATGCCCCTCTCCAACCTGTTT

200 (915)
GCCAACGCTGTGCTGAGGGCTCAGCACCTCCACCTCCTGGCTGCTGAGAC

250 (1407)
ATACAAAGAGATTGAACGCACCTATATTCCGGAGGACCAGAGGTACACCA

300 (1457)
ACAAAAACTCCCAGGCTGCGTTTTGTTACTCAGAAACCATCCCAGCTCCC

350 (1808)
ACGGGGAAGGATGACGCCCAGCAGAAGTCAGACATGGAGCTGCCTCGGTT

400 (1858)
TTCACTGGTTCTCATCCAGTCCTGGCTCACCCCCGTGCAATACCTAAGCA

450 (1908)
AGGTGTTCACGAACAACTTGGTTTTTGGCACCTCAGACAGAGTGTTTGAG

500 (1958)
AAACTAAAGGACCTGGAAGAAGGGATCCAAGCCCTGATGAGGGAGCTGGA

550 (3074)
GGACCGCAGCCCGCGGGGCCCGCAGCTCCTCAGACCCACCTACGACAAGT

600 (3124)
TCGACATCCACCTGCGCAACGAGGACGCCCTGCTGAAGAACTACGGCCTG

650 (3174)
CTGTCCTGCTTCAAGAAGGATCTGCACAAGGTGGAGACCTACCTG

Figure 2.11 - The nucleotide sequence of cDNA produced from chicken medio-basal hypothalamus (MBH) RNA. Bold numbers above the nucleotides relate (last digit aligned with the corresponding nucleotide) to exon nucleotide sequence. The numbers in brackets are the nucleotide sequence of the chicken growth hormone encoding gene (taken from the nucleotide sequence of Tanaka et al, 1992).

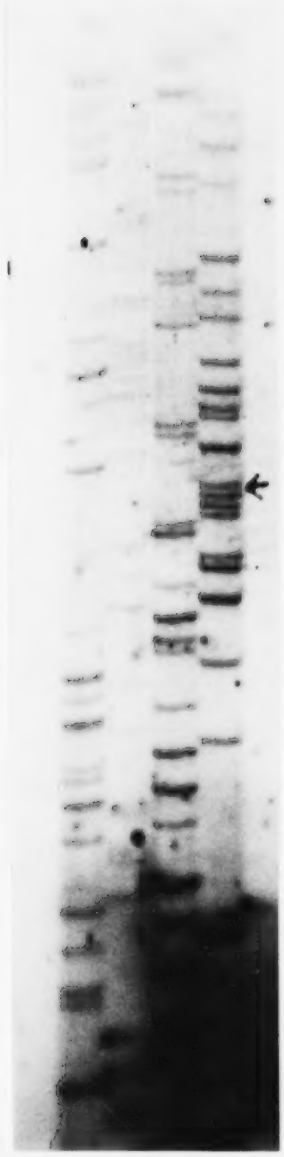

Figure 2.12 - Autoradiograph of the cDNA sequence of a PCR product generated from medio-basal hypothalamic RNA. cDNA was sequenced by a modified dideoxy cycle sequencing method, electrophoresed on 6% polyacrylamide gels and stained with silver nitrate.

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CHAPTER 3. ECTOPIC EXPRESSION AND TRANSLATION OF GROWTH HORMONE AND ITS RECEPTOR IN CHICKEN IMMUNE TISSUES

3.1. Introduction

Regulatory interactions operating between the neuroendocrine axis and the immune system are well established (Weigent & Blalock, 1987; Blalock, 1989; Gilbert and Payan, 1991). Growth hormone (GH) is secreted *in vivo* by the pituitary gland and several lines of evidence suggest that it has endocrine effects on the immune system. Reports have appeared on the positive influence of GH in the development, maturation and function of the immune system. These studies, using hypophysectomized rats and GH-deficient Snell and Ames dwarf mice, have identified defects in both humoral and cell-mediated immunity, including reduced antibody synthesis and delayed allogenic graft rejection (Pierpaoli et al, 1969; Fabris et al, 1971). GH plays a crucial role in both the development of the thymus gland, and its production of hormones such as thymulin. Pituitary deficient animals have small thymus glands (Roth et al, 1984) and exogenous treatment with GH restores deteriorated thymic structure in dwarf mice and aged rats (Pierpaoli et al, 1969; Kelley et al, 1986; Berczi et al, 1991). Similarly, low thymulin levels present in GH deficiency can be improved with GH therapy (Goff et al, 1987; Mocchegiani et al, 1990; Goya et al, 1992; Mocchegiani et al, 1992; Goya et al, 1993). Growth hormone may also be necessary for the normal growth and maintenance of the spleen and bursa of Fabricius, as GH enhanced organ weight and lymphocyte populations in these tissues (Scanes et al, 1990; Haddad and Marshaly, 1991). Improved wound healing (Lynch et al, 1989; Steefos and Janssen, 1992) and reduced tumour metastasis (Torosian and Donoway, 1991) may also be attributable to GH administration. In addition, GH binding sites have been identified on cells belonging to the immune system such as thymocytes, lymphocytes and thymic epithelial cells (Lesniak et al, 1974; Arrenbrecht, 1974; Stewart et al, 1983; Kiess and Butenandt, 1985; Ban et al, 1991), and accordingly GH stimulates proliferation of thymocytes (Berczi et al, 1991; Goya et al, 1992) and lymphocytes (Geffner et al, 1990; Mercola et al, 1981; Suzuki et al, 1990). Furthermore, GH has important regulatory actions in phagocytosis,

acting as a chemoattractant for phagocytic cells (Wiedermann et al, 1992; Wiedermann et al, 1993) and priming macrophages for production of superoxide anion (O_2^-), an important agent in phagocytosis (Edwards et al, 1988; Edwards et al, 1992; Kelley, 1991).

In addition to these endocrine roles of pituitary GH in immune function, the immune system produces GH-like peptides that may also regulate immune function in an autocrine or paracrine fashion. For instance, the synthesis of GH-like mRNA and secretion of GH-like molecules by rodent and human lymphocytes *in vitro* has been reported in a number of studies (Weigent et al, 1988; Hattori et al, 1990; Kao et al, 1992). This GH-like immunoreactivity appears to be active in the immune response as it is released from leukocytes *in vivo* following antigenic challenge (Baxter et al, 1991), and stimulates lymphocyte proliferation (Weigent et al, 1991a). However, it is not clear if the GH molecules arise from the authentic GH gene or a GH-related gene in immune cells.

In the chicken, GH has also been reported to stimulate many aspects of immune function (Marsh, 1992). Treatment of hypophysectomised birds with cGH restores the growth of primary lymphoid organs, and protects against age-associated bursal atrophy (Scanes et al, 1990). The depressed humoral immune response of sex-linked dwarf chickens can also be improved with bGH infusion (Marsh et al, 1984). More recently, Haddad and Mashaly (1991) demonstrated that exogenous cGH treatment increased natural killer (NK) cell activity in chickens. Marsh et al (1992) also found increased numbers of CD4+ and CD8+ cells in response to exogenous GH. It is not known, however, if these are direct effects of GH on immune cells or if GH is produced by immune tissues of the domestic fowl.

The chemistry, synthesis and release of pituitary GH in birds is well documented (Harvey et al, 1991; Scanes et al, 1993) although it is unknown if GH is produced ectopically in this species. An ectopic site of GH production is, however, indicated by the presence of low concentrations of GH in the plasma of hypophysectomized chickens (Harvey et al, 1987; Lazarus and Scanes, 1988). The possibility that the GH gene may be expressed in the lymphoid tissues of chickens was therefore investigated, especially as chickens possess a lymphoid organ, the bursa of

Fabricius, that is unique to birds. In addition, the possibility that GH may have direct actions on avian immune tissues was assessed by determination of GH receptor gene expression in the same lymphoid organs.

3.2. Materials and Methods

Tissues

Spleens, bursa, thymus, liver and pituitaries were rapidly dissected from adult White Leghorn chickens and immediately frozen in liquid nitrogen and stored at -70°C prior to analysis.

Radioimmunoassay (RIA)

The presence of GH-ir in immune tissues was first investigated by radioimmunoassay. Tissues were homogenized (1g/10ml) in RIA buffer (0.04M $NaPO_4$, 0.15M NaCl, 0.1% sodium azide, 0.01M EDTA, 0.5% ovalbumin; pH 7.0) and centrifuged at 2000 x g at 4°C for 30 min. Supernatants were boiled for 10 min to remove endogenous enzyme activity, and cGH was measured in duplicate using a double antibody RIA technique (Harvey & Scanes, 1977). Briefly, cGH was iodinated by the lactoperoxidase method (McIntyre et al, 1974). The reaction mixture contained 5ug hormone, 1mCi [^{125}I]iodine (Amersham, Mississauga, ON, Canada), 10ug lactoperoxidase (0.35 units, Sigma Chemical Co, St.Louis, MO, USA) and 10ul 1.0mM hydrogen peroxide. The reaction was terminated after 2 min by dilution with 0.5ml 0.05M sodium phosphate (pH 7) and labelled hormone had a specific activity of 102.5+/-5.3 (n=10) uCi/ug. Growth hormone was measured in a sample volume of 200ul and a final incubation volume of 400ul. Following a pre-incubation period of the sample with the primary antiserum (rabbit anti-cGH; final dilution 1:500) at 4°C for 24h, tracer (^{125}I-labelled cGH; 10000 c.p.m.) was added and incubated for 24h at 4°C. Precipitating antiserum (donkey anti-rabbit Ig; Wellcome Laboratories) at a final dilution of 1:160 was then added and incubated for a further 24h at 4°C. The assay tubes were then centrifuged at 2000 x g at 4°C for 30 min, the supernatants discarded and the radioactivity in the precipitate was counted using an LKB gammamaster

(Wallac, Turku, Finland). The IC_{50} was 3.67+/-0.37 ng/tube and 10% inhibition, 1.23+/-0.19 ng/tube. Intra-assay and inter-assay coefficient of variation were 6.4 and 17.75% respectively (Harvey and Scanes, 1977).

Immunocytochemistry

The location of GH secreting neurons in the immune tissues was investigated using immunocytochemistry. Sodium pentobarbitol anesthetized adult male chickens were perfused with approximately 500ml phosphate buffered saline (PBS: 137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.7H_2O$; 1.4mM KH_2PO_4) and then with an equal volume of Bouin's solution. Pituitary glands and immune tissues were dissected and post fixed in Bouin's at 4°C overnight. Tissues were dehydrated through a series of alcohols (50%, 70%, 80%, 90%, 100% respectively), cleared in hemo-de (Fisher Scientific, Ottawa, ON, Canada) and embedded in paraplast (Sigma Chemical Co, St.Louis, MO, USA). Sagittal sections (10μm) were cut using a microtome and mounted on to gelatin (0.5%), chromium potassium sulphate (0.05%) coated slides. Immunocytochemical staining was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al, 1981) with commercial reagents (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Briefly, sections were incubated with a specific rabbit polyclonal antisera raised against chicken (c) GH (Harvey & Scanes, 1977; Tai & Chadwick, 1977) diluted 1:5000- 1:8000 in PBS containing 1% normal rabbit serum (NRS) for 18h at room temperature, followed by two 10 min washes in PBS. Sections were then incubated in biotinylated goat anti-rabbit IgG (1:500) for 2h followed by two 10 min washes in PBS and treatment with ABC reagent for 1h. Staining was visualised using the chromagenic substrate diaminobenzidine tetrahydrochloride (DAB, 0.06%, Sigma). The specificity of staining was determined by preabsorbing the cGH antiserum with recombinant cGH (Agen, Thousand Oaks, CA, USA; 1mg/ml) for 1h prior to incubation with sections. The specificity of staining was also determined by incubating the sections with pre-immune rabbit serum rather than the primary antibody. The sections were also incubated with a panel of monoclonal antibodies raised against lymphoid cell markers, which served as a positive control for the fixation of the tissue sections.

Western Analysis

To investigate the presence of GH-ir in immune tissues of the chicken, 1g of frozen tissue (spleen, bursa, thymus and pituitary) was homogenized in 10ml homogenization buffer (1% sodium doedcyl sulphate (SDS); 1mM phenylmethylsulphonylfluoride (PMSF); 10ug/ml aprotinin) using a Brinkman Polytron homogenizer (Brinkman Instruments, IL, USA). The homogenates were centrifuged at 2000 x g for 5 min at 4°C and the supernatants collected. Protein content was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard and coomassie blue as indicator. Samples containing 30μg protein were diluted 1:1 with loading buffer (0.06M Tris.Cl, pH 6.8;10% glycerol; 2% SDS; 8% 2-β-mercaptoethanol; 0.01% bromophenol blue) and heated to 55°C for 15 min prior to electrophoretic seraration on 15% SDS-polyacrylamide gels. After seperation the gels were equilibrated in transfer buffer (25mM Tris; 192mM glycine; 20% methanol) for 30-60 min and electrotransferred to immobilon PVDF membranes (Millipore, Bedford, MA, USA) at 30 V for 4h at 4°C. The membranes were blocked in 5% non-fat dry milk dissolved in tris buffered saline (TBS: 25mM Tris.HCl, pH 7.5; 0.5M NaCl) for 1h at room temperature. GH-ir was detected by incubation of membranes with a rabbit polyclonal antibody raised against cGH (Harvey & Scanes, 1977; Tai & Chadwick, 1977) (1:2000) for 4h at room temperature. Antibody binding for cGH was visualised using an anti-rabbit IgG horseradish peroxidase conjugate (Amersham, Mississauga, ON, Canada) diluted 1:2000 in TBS/5% non-fat dry milk. Alternatively, the membranes were incubated with concanavalin A horseradish peroxidase conjugate (Con-A-HRP: 4ug/ml;Sigma) for 4 hrs. Immunoreactive or glycosylated bands were developed by incubating membranes in ICL reagent (Amersham) for 1 min and exposing membranes to Kodak X-AR film (Kodak, Rochester, NY, USA).

Ribonucleic acid (RNA) extraction

The possible expression of the GH gene and the GH receptor gene in immune tissues of the chicken was investigated using the PCR. Total cellular RNA was extracted from immune tissues, livers and pituitaries by Polytron homogenization (Brinkman Instruments) in 5.5M guanidium thiocyanate containing 25mM sodium

citrate, 0.5% (w/v) sodium lauryl sarcosine (pH 7.0) and 0.2% 2-β-mercaptoethanol and collected after isopycnic ultra centrifugation (125,000 x g at 22°C for 24 hr) through a gradient of Caesium trifluoroacetic acid (Pharmacia fine chemicals, Uppsala, Sweden; Density 1.51±0.01g/ml) containing 0.1M EDTA (pH 7.0). The RNA was resuspended in diethylpyrocarbonate (DEPC) treated water and quantified spectrophotometrically at 260nm. In all samples optical density ratios were between 1.8 and 2.1. Purity of RNA was also determined by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide (Maniatis et al, 1982).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA extracted from tissues was then used for complementary deoxyribonucleic acid (cDNA) synthesis with superscript reverse transcriptase (100U; BRL, Burlington, ON, Canada) primed with 50pmol antisense primer 5'GCCTCAGATGGTGCAGTTGCTCTCTCCGAA3' (for GH) or 100pmol oligodeoxythymidine primer (Boehringer Mannheim, Laval, QU, Canada) in the presence of 5X-HRT buffer (BRL) and 10mM of each deoxynucleotide. The reaction mixture was incubated for 1h at 37°C and 30 min at 42°C. A negative control was performed for the first strand synthesis which contained all the reagents except RNA. An aliquot (0.05%) of the first strand synthesis reactions was then amplified for 30 cycles in a mixture containing 1X PCR buffer (80mM KCl; 16mM Tris.HCl, pH 8.4; 1.5mM $MgCl_2$; 0.1% Triton X-100), 200μM of each deoxynucleotide, 15pmol of both 5' oligomer sense and 3' oligomer antisense primers and *Thermus aquaticus* (*Taq*) DNA polymerase (5U; Promega, Madison, WI, USA). Different types of amplification were carried out; amplification of the hormone cDNA was performed with immune tissues and pituitary using primers: CRE1 5'CGTTCAAGCAACACCTGAGCAACTCTCCCG3' and CRE2 5'GCCTCAGATGGTGCAGTTGCTCTCTCCGAA3' whose positions are depicted in figure 3.5. These primers were based on the known sequence of the cGH cDNA (Lamb et al, 1988). Amplification of the cGH receptor cDNA from immune tissues and liver used primers KHU1 (5'CCTCGATTTGGATACCATATTGTGTTAAGC3') and KHU2 (5'CTGTTACGGCCAGCCCACACACTCCGAAG3'). The primers were

based on the known sequence of a portion of the cGH receptor transcript coding for the extracellular domain (Burnside et al, 1991). The reverse transcribed RNA was also amplified in the presence of primers KHU3 (5'CTGCGGCCGCAGGACCAGTTCCAAAGATTAA3') and KHU4 (5'AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC3') coding for a portion of the intracellular domain of the receptor (Burnside et al, 1991). The mixture was overlayed with mineral oil (v/v), and amplifications were carried out for 30 cycles at 94°C for 1 min (denaturing), 50°C for 1 min (annealing) and 72°C for 2 mins (extension), with a final extension (72°C for 10 min) in a genetic thermal cycler (MJ research, Watertown, MA, USA). The PCR products were resolved by 1.5% (w/v) agarose gel electrophoresis and visualised by ethidium bromide staining.

Restriction Endonuclease digestion

A sample of the GH cDNA produced by the RT-PCR was further characterized by restriction endonuclease digestion with *BamHI* or *RSaI* (5U/μg DNA; BRL) for 2h at 37°C, to determine if the cDNA contained the retriction sites present in the pituitary GH cDNA. GH receptor cDNA was restriction endonuclease digested for 2h at 37°C with *BamHI* and *NcoI* or *EcoRI* and *HaeIII* for known cleavage sites in the regions coding for the extracellular and intracellular domains of the receptor respectively. The restriction endonuclease digestion reactions along with an untreated sample and size standards were then fractionated by 1.5% (w/v) agarose gel electrophoresis, stained and examined as described above.

cDNA probe synthesis

The identity of the GH-like PCR products was also investigated by Southern analysis. A cDNA probe constructed from an 803 bp fragment of the cGH cDNA sequence inserted into the plasmid vector PUC119 was kindly donated by Dr Douglas Foster (Dept. Animal Science, University of Minnesota). The Plasmid PUC119 was linearized by restriction enzyme digestion with *EcoRI* (5U/μg DNA; BRL) and the cDNA fragment was isolated from the vector DNA by electrophoretic seperation on 1.5% agarose gels. The cDNA was then purified using Geneclean II (BioCan

Scientific, Missisauga, ON, Canada) and restriction endonuclease digested with *BstXI* and *HaeII* (5U/μg DNA; BRL) to produce a 613 bp fragment which did not contain the oligonucleotide primer sequences used in RT-PCR. The cDNA probe was then radiolabelled by the random priming method (BRL) in which the cDNA is incubated with short primers coding for a wide range of nucleotide sequences (Maniatis et al, 1982). Template cDNA was incubated with nucleotide mix (dATP, dGTP and dTTP; 0.5mM each), ^{32}P labelled dCTP (Dupont, Mississauga, ON, Canada), and random hexamer primers and synthesis of radiolabelled template was carried out by Klenow fragment (DNA polymerase 1; 3U). Radiolabelled template was purified from excess nucleotides and enzyme by Nuctrap push colums (Stratagene, La Jolla, CA, USA) and resuspended in double distilled water prior to incubation with membranes.

Southern Blot Analysis

To determine if PCR products were homologous to cGH the PCR reactions were electrophoresed through a 1.5% (w/v) agarose gel and the cDNA was transfered to Hybond N nylon membrane (Amersham) by capillarity, prior to baking membranes at 80°C for 2h under vacuum. Membranes were prehybridized for 3h at 42°C in 50% formamide containing, 5X SSC (1X SSC:0.15M NaCl; 0.015M sodium citrate, pH 7.2), 5X Denhardts, 1% SDS, and 50μg/ml Salmon sperm DNA (Sigma) and hybridized under the same conditions for 18h in the presence of 10% Dextran Sulphate and newly synthesized probe. Following hybridization the membranes were washed sequentially at 60°C in decreasing concentrations of SSC. 15 min washes in 2X, 1X, 0.5X, and 0.1X SSC containing 0.1% SDS were performed. Membranes were exposed to Kodak X-AR film (Kodak) between intensifying screens at -70°C and autoradiographed.

PCR sequencing

Further confirmation that PCR products were produced from authentic GH mRNA in immune tissues was obtained by sequence analysis of the PCR products. PCR reaction products were electrophoresed in 1.5% (w/v) agarose ethidium bromide stained minigels (Maniatis et al, 1982). The appropriate size ethidium staining DNA

band (774 bp) was excised from the gel and purified from excess nucleotides and agarose using Geneclean II (BioCan Scientific), according to manufacturers instructions. The fragment was then resuspended in double distilled water prior to sequencing. PCR fragments were cycle sequenced (both strands) by a modified cycle dideoxy chain termination method (Sanger et al, 1977). Briefly, 2pmol template DNA was added to a mixture of 5X sequencing buffer (250mM Tris.HCl pH9; 10mM $MgCl_2$: Promega), 5'- oligomer sense (CGTTCAAGCAACACCTGAGCAACTCTCCCG) or 3'-oligomer antisense (GCCTCAGATGGTGCAGTTGCTCTCTCCGAA) primer (4.5pmol), sequencing grade *Taq* DNA polymerase (Promega) and one of the 4 silver sequence deoxy/dideoxy nucleotides (d/ddATP, d/ddCTP, d/ddGTP, or d/ddTTP). The primers were based on the published sequence of GH cDNA (Lamb et al, 1988). The mixture was overlayed with mineral oil (v/v) and heat denatured at 95°C for 2 min before 55 cycles of 95°C for 30 sec (denaturing step) and 70°C for 30 sec (annealing /extension step) in a genetic thermal cycler (MJ Research). The reaction was terminated by addition of DNA sequencing stop solution (10mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanol). cDNA from each tissue was sequenced 5-6 times to increase fidelity of results. Sequenced products were heated at 70°C for 2 min prior to loading on 6% (v/v) acrylamide/bis-acrylamide gels (19:1), containing 1X TBE (0.089M Tris; 0.089M Boric acid; 20mM EDTA pH8) and electrophoresed at 1800V for 1.5 to 2.5h. After electrophoresis the gel plates were separated and the gel fixed in 10% glacial acetic acid, stained with silver nitrate (1g/L) solution, containing 0.05% formaldehyde, and developed with chilled (10 - 12°C) Na_2CO_3 solution (30g/L), containing sodium thiosulphate (2mg/L) and formaldehyde (0.05%). The developing reaction was terminated by addition of 10% glacial acetic acid (v/v), and gels were air dried, prior to exposure to EDF film (Kodak) for 10 to 15 sec.

3.3. Results

Immunoreactive cGH in immune tissues

The presence of immunoreactive GH-like material in immune tissues was investigated using radioimmunoassay. Serial dilutions of boiled crude saline extracts

of the spleen, thymus, bursa and pituitary gland of the chicken displaced the binding of ^{125}I-GH tracer to GH antisera in a manner parallel to that of the standard (fig 3.1). The amount of immunoreactive GH material in these immune tissues was, however, consistently less than that found in the pituitary gland (Table 3.1), as this tissue was able to completely supress tracer binding to the GH antisera. The immunoreactive GH in immune tissues was greater than that present in muscle, liver or duodenum which showed no apparent GH immunoreactivity (<0.1 ng/ml).

Immunocytochemistry

In studies using unadsorbed antisera to cGH, a peroxidase reaction product was observed in numerous cells distributed evenly througout the caudal lobe of the chicken anterior pituitary gland (fig 3.2). Few cells within the cephalic lobe were stained using this method. After preadsorption with cGH (1mg/ml), a complete loss of immunostaining was achieved indicating the specificity of the technique (data not shown). However, sections of bursa, thymus, and spleen appeared negative for GH-ir as no peroxidase reaction product could be detected. These sections did, however, stain when incubated with a panel of monoclonal antibodies raised against lymphoid cell markers (data not shown).

Western Analysis

Figure 3.3 shows the results obtained when extracts of pituitary gland and immune tissues were analysed by SDS-PAGE and western blot employing a polyclonal antibody raised against cGH. Several immunoreactive cGH-like bands were detected in pituitary gland with apparent molecular weights of 16, 22, 26, and 44 kDa when compared with prestained molecular weight standards. In contrast a single GH immunoreactive band of approximately 26kDa was identified in bursa and thymus which corresponded with the majority of the GH immunoreactivity in the chicken pituitary gland. In addition spleen tissue extracts contained a faint GH immunoreactive band of 26kDa and a more intense higher molecular weight band of 44kDa. Staining was abolished by immunoadsorption of the primary antibody with

rcGH (fig 3.3b) prior to its incubation with membranes, and no staining was observed when the primary antibody was replaced with pre-immune serum (fig 3.3c).

Lectin blots of immune tissue extracts with Con A-HRP staining indicated carbohydrate moeities in high molecular weight proteins but no glucose or high mannose carbohydrate chains in the 26kDa or 44kDa region (fig 3.4).

Expression of the GH gene in immune tissues of the chicken

To characterize the mRNA produced by chicken immune tissues GH specific sequences were amplified by reverse transcription and PCR using two oligonucleotide primers specific for chicken pituitary GH (fig 3.5). RNA was used as a template for selective first strand cDNA synthesis with an antisense oligonucleotide primer complementary to the 3' end of exon 5 of cGH mRNA. This cDNA was then introduced into a PCR with a second sense oligonucleotide primer coding for 30 bp of exon 1 of the cGH sequence, located 20 bases from the exon start site. This PCR reaction amplified a cDNA of the appropriate size (689bp) in all immune tissues studied which was of similar size to that amplified from chicken pituitary gland (fig 3.6a). No bands were observed in cDNA amplified from muscle or liver, and likewise, exclusion of RNA or superscript enzyme from the initial RT reaction did not produce any cDNA (data not shown). Restriction endonuclease mapping of the cGH gene has identified cleavage sites for the enzymes *BamHI* and *RsaI*. Digestion of the cDNA produced from bursa, spleen and thymus with *BamHI* produced fragments of the expected sizes of 465 and 224bp as detailed in figure 3.6b. *RsaI* digestion of these PCR products also produced bands of expected size, 237bp and 452bp (data not shown).

Southern Analysis

To confirm that the cDNA generated from bursa, spleen and thymus shared sequence homology with pituitary GH cDNA southern analysis was performed. A radiolabelled cGH cDNA probe was found to hybridize with PCR fragments amplified from each of the immune tissues studied. Hybridization occured with a single moiety of approximately 689bp in each of the immune tissues, and also with cDNA amplified

from pituitary gland (fig 3.7). This hybridization appears to be specific as no signal was detected in samples from muscle and liver.

PCR sequencing

The sequence identity of cDNA produced from chicken immune tissues using specific primers for the cGH sequence was investigated using a modified cycle dideoxy chain termination method. Comparison of banding patterns on silver stained acrylamide gels indicated the cDNA sequence of bursa, spleen and thymus PCR products closely resembled that of pituitary cDNA (fig 3.8). The sequence of 613 bp of the bursa PCR product was determined and found to bear a 100% homology to region 49 to 662 of the exon coding regon of the cGH gene sequence (fig 3.9). In addition spleen and thymus cDNA also bore a 100% homology with the cGH gene sequence after sequencing 593 and 604 bp of their length respectively.

Expression of the cGH Receptor in Lymphoid Tissues

Chicken GH receptor expression in lymphoid tissues was examined by PCR after reverse transcription of total RNA with oligodeoxythymine primer. In the presence of 3' and 5'-oligonucleotide primers for the extracellular and transmembrane domains of the chicken liver receptor cDNA, cDNA synthesized from chicken thymus and spleen was amplified by PCR and stained with ethidium bromide. A single band of approximately 500 bp in size comigrated with the 499 bp PCR product amplified from chicken liver cDNA (fig 3.10a). When immune tissue cDNA was digested with *BamHI*, two bands of approximately 325 bp and 175 bp were observed, as would be expected for digestion of the chicken GHR cDNA fragment (fig 3.10b). Digestion of the cDNA with *NcoI* also produced fragments of the expected size 350 bp and 150 bp (data not shown). Amplification of cDNA with cGHR 3' and 5'-oligonucleotide primers for the intracellular domain of the receptor revealed a single electrophoretic band of approximately 800 bp (fig 3.10a). As expected, *HaeIII* digestion of these immune tissue PCR fragments produced cDNA of approximately 469 bp and 337 bp (fig 3.10b). Moieties of predicted size were also generated following digestion of cDNA with *EcoRI* (530 bp and 275 bp) (data not shown). The results of both sets of

amplifications and digestions mirrored those observed with hepatic cDNA (fig 3.10a and 3.10b). In both PCR reactions, these fragments were not generated when RNA was not transcribed by superscript or was absent.

3.4. Discussion

Both *in vitro* and *in vivo* studies have implicated pituitary GH as an important regulator of immune function. In the present study we have presented immunological and molecular evidence that GH is produced by normal lymphoid tissues of the domestic fowl. The expression of the GH receptor gene in these tissues also suggests that GH may have local actions and behave as an autocrine or paracrine agent.

Using SDS-PAGE and immunoblotting with an polyclonal antibody raised against cGH, we identified a protein with a molecular size of approximately 26 kDa, identical to the major fraction of GH-ir in the pituitary gland, in all of the immune tissues studied. In addition a 44 kDa GH-ir band appeared to be the major GH species in chicken spleen, but was not present in either bursa or thymus. Heterogeneity of pituitary GH has similarly been demonstrated in several species (Aramburo et al, 1990; Baumann, 1991). Indeed, multiple forms of GH-ir have also been described in rat and human lymphocytes, which may represent oligomers or GH linked to plasma proteins (Weigent et al,1987; Hattori et al, 1990). Our results are consistent with this observation, and the 44 kDa GH-ir protein present in chicken spleen may represent a dimer of monomeric GH. As this GH-ir species was present only in spleen it also suggests a heterogeneity within immune tissues with respect to their ability to produce ir-GH. These GH-ir proteins did not contain glucose or high mannose carbohydrate moieties based on analysis of tissue homogenates on lectin blots stained with a Con-A horseradish peroxidase conjugate. However, this does not rule out the possibility of glycosylation by some other carbohydrate group. The GH-like proteins in chicken immune tissues had a cross-reactivity with the antisera raised against pituitary GH in RIA, that was parallel with that of purified chicken GH. The spleen extract, however, exhibited a different pattern of displacement. The dose response inhibition curve for spleen extract was similar to that of the standard at low tissue extract levels. The lack of displacement observed with the spleen at higher tissue extract

concentrations, may be partially due to the presence of increased levels or concentration of GH binding proteins compared to the other tissues studied. Although plausible, little data exists to support this argument (Baumann, 1993). Alternatively, it may be that the dimeric GH variant found in the spleen in this study, is not as effective in displacing the tracer from the antisera as the GH monomer, thus interfering with the assay. The tissue extracts used in RIA were boiled prior to analysis to remove any endogenous enzyme activity, however, this may have also resulted in a change in conformation of the protein under investigation. This may also also account for the inability of the spleen extract to displace binding in this study. When the same antibody was used for immunocytochemistry no GH-ir could be detected on paraffin sections of immune tissues, even though strong immuno-staining was observed in pituitary gland. These immune tissue sections were, however, found to stain with a panel of monoclonal antibodies raised against lymphoid cell markers. These markers thus served as a positive control to suggest the tissues were adequately fixed, although fixation may have compromised the GH immunogenicity of the same sections. It is, however, also possible that immune GH and pituitary GH have some differences in epitope structure that are not revealed by RIA. These differences may be due to differential post-translational processing, as PCR experiments using primers located in the coding region of the pituitary GH cDNA sequence, amplified only a single GH cDNA from all immune tissues studied. This finding further supports the assumption that the 44 kDa GH-ir species in chicken spleen is a dimeric form of GH and not a gene variant.

The GH RNA isolated from chicken immune tissues appeared to be similar to pituitary GH RNA, as the cDNA generated from these tissues by RT-PCR, hybridized with a specific GH cDNA after Southern transfer and contained sequences recognised by the restriction endonucleases *Bam HI* and *RSa I*. Moreover, sequence analysis of the cDNA revealed a complete homology with pituitary GH cDNA implying that the same gene is expressed in both the pituitary gland and immune tissues. Taken together these data are consistent with the recent demonstration of production of a molecule identical to pituitary GH by lymphoid cells in the rat (Weigent et al, 1991a; Weigent et al, 1991b; Baxter et al, 1991) and human (Hattori et

al, 1990) immune systems as well as by human lymphocyte cell lines (Kao et al, 1992; Yang et al, 1993). In these systems, however, the GH mRNA was present in amounts sufficient to be detected by Northern and slot blot analysis. This may be due to the use of isolated lymphocytes in culture medium, whereas the present study examined entire immune organs and utilized the more sensitive technique of RT-PCR. Interestingly, another study by Binder et al (1994) similarly failed to demonstrate GH mRNA in immune organs of adult rats, and these authors suggested that GH expression was transient in nature and occured only during the period of development of the immune system. As GH mRNA did not appear within 1h of culturing lymphocytes, and ir-GH could be radiolabelled with tritiated amino acids and its production blocked by prior incubation with cyclohexamide (Weigent et al, 1988), this indicates the *de novo* synthesis of both GH mRNA and protein. The possibility that the gene for GH is under negative control in leukocytes and is stimulated only in response to antigenic challenge was therefore suggested (Weigent et al, 1988; Baxter et al, 1991), and may explain the failure of Binder et al (1994) to detect GH mRNA in adult immune tissues. The demonstration of GH expression and translation in immune tissues of the adult chicken under unstimulated conditions *in vivo*, however, argues against these points. The specificity of the highly sensitive RT-PCR technique was ensured by taking a number of precautions. Contamination of PCR reactions by cDNA was controlled by performing RT in the absence of RNA or enzyme, and a chicken muscle sample that was negative for GH cDNA was used as a control in all amplification reactions. Contamination by genomic DNA is unlikely because the primers used in the PCR were designed from two different exons, and larger fragments containing intron sequences would have been amplified from genomic DNA. The sensitivity of the reaction was also demonstrated by using pituitary RNA as a positive control in all experiments. The expression and translation of the GH gene therefore appears to be a normal function of chicken immune tissues.

Recently expression of the pituitary specific transcription factor, Pit-1 has been demonstrated in lymphoid tissues of the rat (Delhase et al, 1993). It has therefore been suggested that Pit-1 may control the expression of the GH gene in these tissues. The upstream region of the cGH gene contains a nucleotide sequence which is highly

homologous to the antisense strand sequence of the proximal binding site for Pit-1 in the promoter region of the rat GH gene (Tanaka et al, 1992). Although this region of the GH gene was not sequenced in the present study, the high homology between the coding region of the gene expressed in immune tissues and the cGH gene suggests that they are the same gene. A similar regulatory mechanism of gene expression involving Pit-1 may, therefore, also be present in immune tissues of the chicken.

The finding that chicken immune tissues are able to express and possibly translate their own GH, suggests that pituitary GH may not be crucial for maintenance of immunological competence. The immunomodulatory role of GH has been documented mostly in pituitary deficient rats and mice which have dramatically lower plasma GH levels. These animals have been found to have reduced antibody synthesis and delayed allogenic graft rejection (Pierpaoli et al, 1969; Fabris et al, 1971). In humans GH may not play such an important role in immune function (Wit et al, 1993), but GH deficiency is associated with reduced natural killer cell activity (Kiess et al, 1986). In rats, it has been demonstrated that antisense oligonucleotides to rat GH message can decrease the production of ir-GH by rat spleen lymphocytes, as well as inhibit the proliferation of the lymphocytes themselves (Weigent et al, 1991a). In addition, GH augments the release of GH-ir from human lymphocytes (Hattori et al, 1990). These reports therefore strongly support a physiological role for GH in immunoregulation and suggest that these effects of GH are directly mediated on immune cells.

Endocrine, paracrine or autocrine effects of GH on immune cells axiomatically imply the presence of GH receptors that mediate signal transduction. The presence of such receptors was indicated in previous studies by the occurence of GH binding sites in human mononuclear cells, IM-9 lymphocytes and thymic epithelail cells (Lesniak et al, 1974; Stewart et al, 1983; Kiess and Butenandt, 1985) with binding affinities comparable with hepatic GH receptors. Although some of these binding sites may reflect lactogenic receptors rather than somatogenic receptors (Dardenne et al, 1989), GH receptor gene expression in human and rat immune tissues (Ban et al, 1991) and in chicken lympoid organs (fig 3.10) indicates that most are likely to be authentic GH receptors. The actions of GH within the immune system may, however, be indirect

and mediated by the local production of growth factors, since the proliferative effect of hGH on rat thymic cells is IGF-I dependent (Geffner et al, 1990)

In summary, the present study indicates that the cGH gene is expressed and translated in the immune tissues of the chicken, in which it may act in an autocrine or paracrine fashion, like many other cytokines present in the immune system.

3.5. Figures

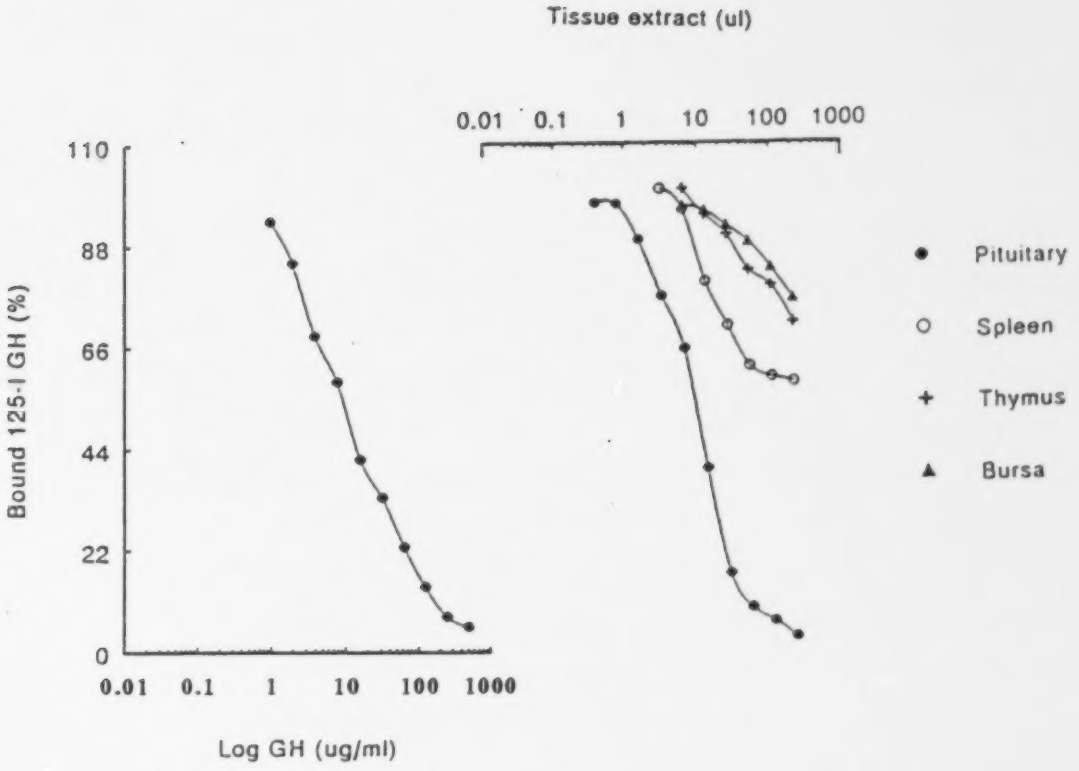

Figure 3.1 - Cross reaction of crude saline homogenates of pituitary, spleen, thymus and bursa with a cGH antisera. Dose response curves for the immune tissue extracts showed parallelism with the standard curve. The slopes of the lines lay within the 95% confidence limits for the GH standard (-0.57 to -0.64).

Table 3.1 - Immunoreactive growth hormone levels in immune tissues of the chicken.

Tissue Extract	ir-GH ng/mg protein
Bursa	0.32
Spleen	3.61
Thymus	0.58
Pituitary	38.09
Muscle	ND
Duodenum	ND
Liver	ND

ir-GH levels as determined by cross-reactivity with GH antisera in a radioimmunoassay; ND, not detectable.

Figure 3.2 - Sagittal section through the chicken pituitary gland demonstrating the location of immunoperoxidase staining after incubation with a rabbit polyclonal antibody raised against cGH.

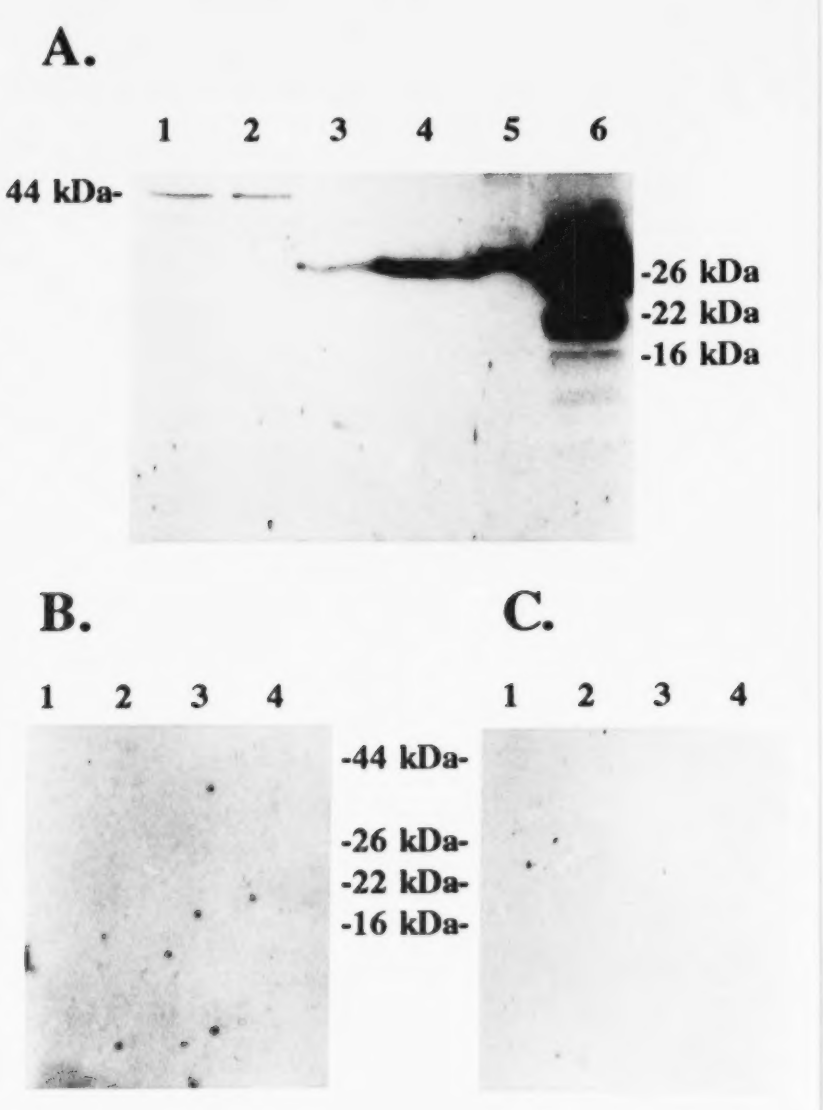

Figure 3.3 - Immunoblot detection of GH in chicken immune tissues following SDS-PAGE on 15% gels, under reducing conditions. Immunoblotting procedures were performed as described using a polyclonal antibody raised against cGH at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1:2000. (A) Stained with α-cGH, Lanes: (1 and 2) Spleen; (3 and 4) Thymus gland; (5) Bursa extract; (6) Pituitary extract. (B) Preabsorption of α-cGH with rcGH (1mg/ml). (C) Incubation with Normal rabbit serum.

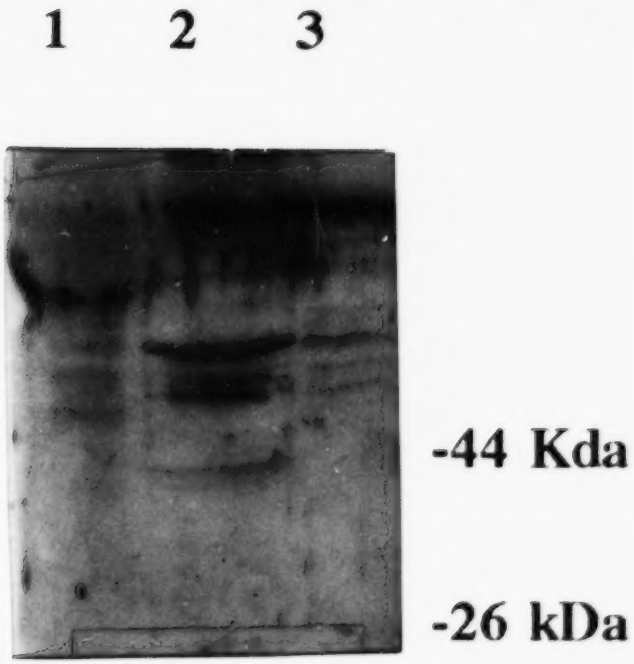

Figure 3.4 - SDS-PAGE and lectin blot analysis of glycosylated proteins in extracts of immune tissues. Bands were detected by incubation with a Con A horseradish peroxidase conjugate (1:1000). Lanes (1) Thymus; (2) Spleen; (3) Bursa.

A.

Primers

cre1: 5' gcctcagatggtgcagttgctctctccgaa 3'

cre2: 5' cgttcaagcaacacctgagcaactctcccg 3'

B.

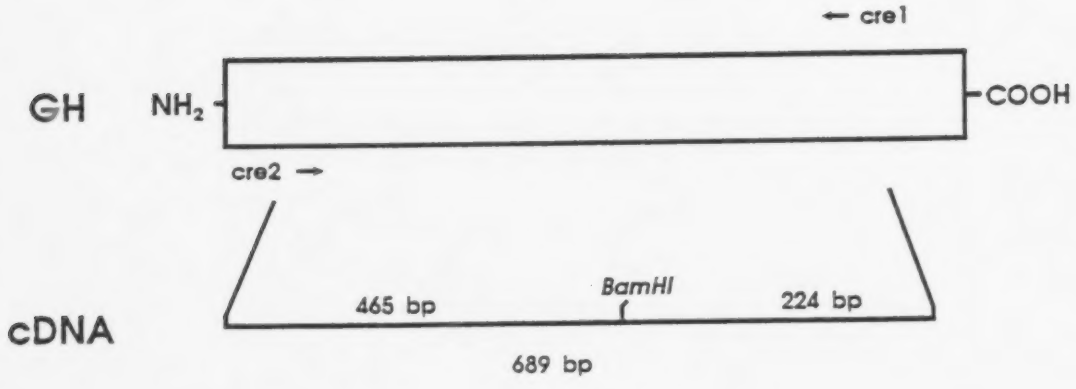

Figure 3.5 - (A) Oligonucleotide primers (CRE 1 and CRE 2) used in the polymerase chain reaction (PCR) to amplify the coding region of the GH gene. (B) Sites of primer annealing and restriction endonuclease digestion site on the GH cDNA.

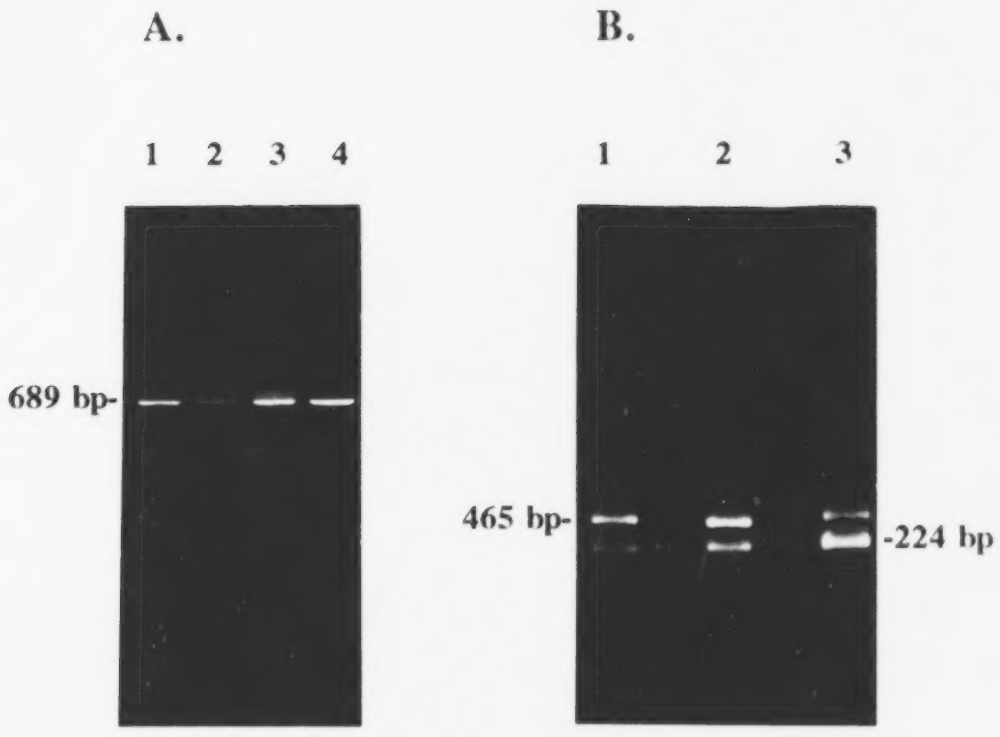

Figure 3.6 - Ethidium bromide stained 1.5% agarose gel through which cDNA was electrophoresed. The size of the fragments was determined by comparison with ΦX174RF DNA/HaeIII size markers. (A) The cDNA was reverse transcribed from total RNA extracted from chicken bursa (lane 1), spleen (lane 2), thymus (lane 3) and pituitary gland (lane 4), and amplified in the presence of oligonucleotide primers CRE1 (exon 1) and CRE2 (exon 5). (B) Bam H1 digests of bursa (lane 1) spleen (lane 2) and thymus (lane 3) cDNA are also shown.

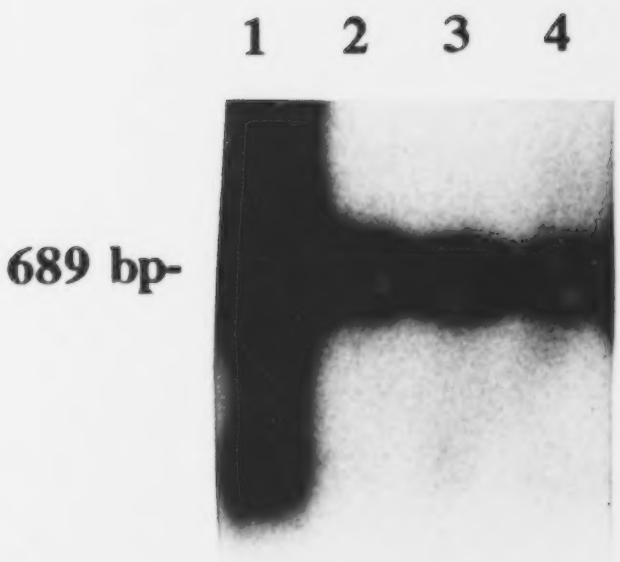

Figure 3.7 - Southern analysis. RT-PCR products amplified from chicken pituitary (lane 1), bursa (lane 2), spleen (lane 3) and thymus (lane 4) RNA were southern transfered onto nylon membranes and analysed using a radiolabelled cGH cDNA as an internal probe.

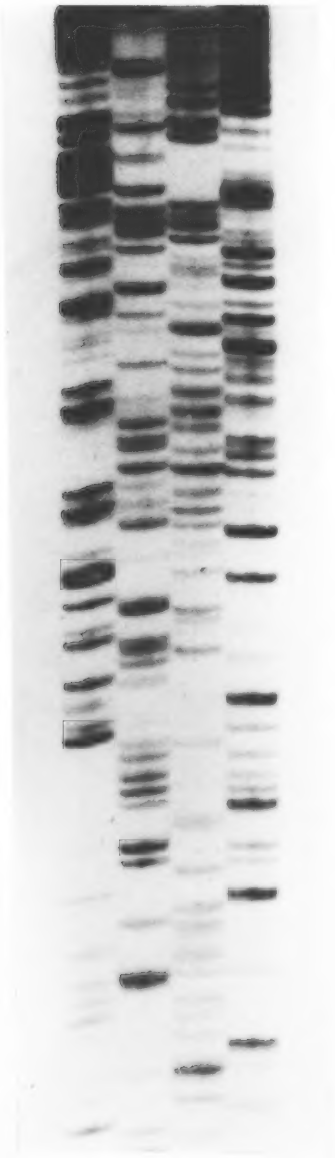

Figure 3.8 - Autoradiograph of the cDNA sequence of a PCR product generated from bursa of Fabricius RNA. cDNA was sequenced by a modified dideoxy cycle sequencing method, electrophoresed on 6% polyacrylamide gels and stained with silver nitrate.

100 (815)
CCAGGCTCGTGGTTTTCTCCTCTCCTCATCGCTGTGGTCACGCTGGGACT

150 (865)
GCCGCAGGAAGCTGCTGCCACCTTCCCTGCCATGCCCCTCTCCAACCTGT

200 (915)
TTGCCAACGCTGTGCTGAGGGCTCAGCACCTCCACCTCCTGGCTGCTGAG

250 (1407)
ACATACAAAGAGATTGAACGCACCTATATTCCGGAGGACCAGAGGTACAC

300 (1457)
CAACAAAAACTCCCAGGCTGCGTTTTGTTACTCAGAAACCATCCCAGCTC

350 (1808)
CCACGGGGAAGGATGACGCCCAGCAGAAGTCAGACATGGAGCTGCCTCGG

400 (1858)
TTTTCACTGGTTCTCATCCAGTCCTGGCTCACCCCCGTGCAATACCTAAG

450 (1908)
CAAGGTGTTCACGAACAACTTGGTTTTTGGCACCTCAGACAGAGTGTTTG

500 (1958)
AGAAACTAAAGGACCTGGAAGAAGGGATCCAAGCCCTGATGAGGGAGCTG

550 (3074)
GAGGACCGCAGCCCGCGGGGCCCGCAGCTCCTCAGACCCACCTACGACAA

600 (3124)
GTTCGACATCCACCTGCGCAACGAGGACGCCCTGCTGAAGAACTACGGCCT

650 (3174)
GCTGTCCTGCTTCAAGAAGCATCTGCACAAGGTGGAGACCTACCTGAAGGT

GATGAAGTGCCG

Figure 3.9 - The nucleotide sequence of cDNA produced from chicken bursa of Fabricius RNA. Numbers above the nucleotides relate (last digit aligned with the corresponding nucleotide) to nucleotide sequence. Bold numbers above the nucleotides relate (last digit aligned with the corresponding nucleotide) to exon nucleotide sequence. The numbers in brackets are the nucleotide sequence of the chicken growth hormone encoding gene (taken from the nucleotide sequence of Tanaka et al, 1992).

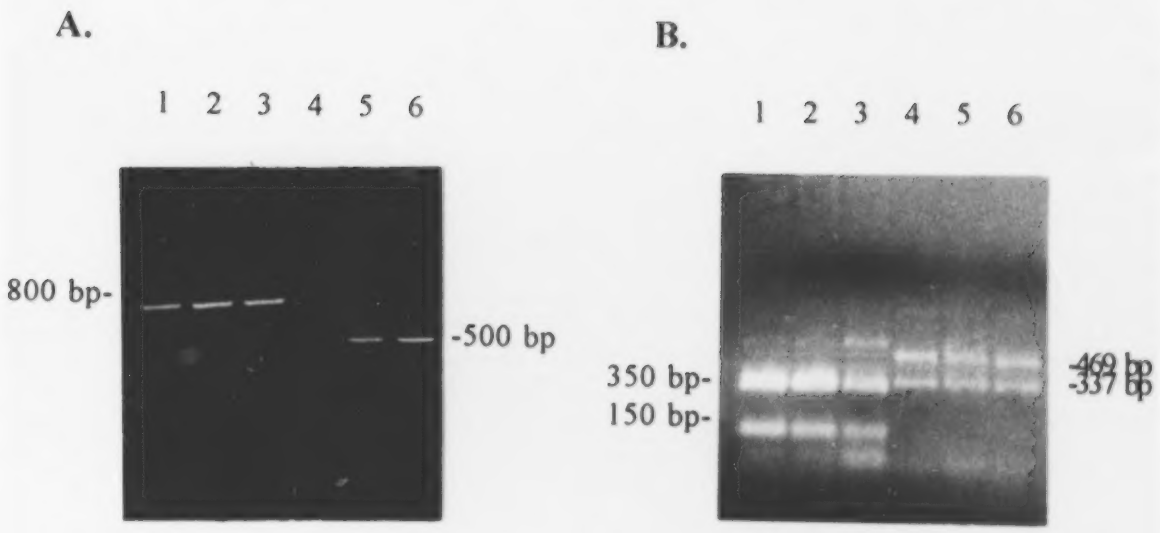

Figure 3.10. - (a) Ethidium bromide stained 1.5% agarose gel through which cDNA was electrophoresed. The cDNA was reverse transcribed from total RNA extracted from adult chicken spleen (lane 1), thymus (lane 2) and liver (lane 3) and amplified in the presence of oligonucleotide primers KHU3 and KHU4 coding for the intracellular domain of the chicken GH receptor. cDNA was also amplified in the presence of oligonucleotide primers KHU1 and KHU2 coding for the extracellular and trans-membrane domains of the GH receptor (spleen, lane 4: thymus, lane 5: liver, lane 6). (b) *HaeIII* digests of cDNA amplified using the oligonucleotide primers for the intracellular domain (spleen, lane 1: thymus, lane 2: liver, lane 3) and *BamHI* digests of spleen (lane 4), thymus (lane 5) and liver (lane 6) cDNA amplified using the extracellular primers are also shown.

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CHAPTER 4. SUMMARY AND CONCLUSIONS

4.1. Summary

Growth hormone is a multifunctional anterior pituitary hormone with well recognised and diverse functions involving both growth promoting and metabolic actions. The presence of GH in ectopic sites has been the subject of several recent investigations, and it has been proposed that GH is present in neural and immune tissues, where it may have functions unrelated to growth (Hojvat et al, 1982; Weigent & Blalock, 1987).

This thesis has examined the expression and translation of GH in neural tissues of the chicken and in addition investigated the expression of components of a GH communication system in lymphoid tissues. It was hypothesized that 1) the GH gene is expressed in these ectopic sites, 2) the GH gene is identical to the GH gene expressed in the chicken pituitary gland, 3) the GH gene is transcribed to immunoreactive protein in these tissues and, 4) the GH receptor is expressed in immune tissues, therefore providing a functional mechanism for GH action in this system.

In Chapter 2, the expression of the GH gene and its possible translation was examined in neural tissues of the chicken. Although previous studies have indicated GH gene expression and the presence of GH-like imunoreactivity in mammalian brain, these studies provided conflicting reports on sites of synthesis and location of immunoreactivity (Gossard et al, 1987; Lechan et al, 1981). The data presented herein are the first to document the co-localization of GH gene expression and GH-ir in hypothalamic tissue and to demonstrate that the coding region of this gene appears to be identical to the GH gene expressed in the pituitary gland. Furthermore, in Chapter 3 the expression of both GH and the GH receptor was demonstrated in chicken immune tissues. The GH gene expressed in these immune tissues shares complete homology with the GH gene expressed in the pituitary gland and may be translated to immunoreactive protein in lymphoid tissues.

4.2. Evolutionary Implications

The presence of this hormone in tissues outside the specialized glandular location of the pituitary raises a number of questions about the origins, regulation and roles of GH. Roth et al (1983), have identified material in unicellular organisms which closely resembles vertebrate messenger molecules and receptors. These immunoreactivities include the hypothalamic and pituitary hormones SRIF, TSH, ACTH, LH and follicle stimulating hormone (FSH). The function of these neuroendocrine elements in microbes is not yet established, but some evidence exists to suggest they may be humoral factors involved in transfer of genetic material (Dunny et al, 1979) and regulation of food intake (Sarkar et al, 1979). It is unclear when GH appears during phylogeny, but studies in invertebrates have identified GH-ir using antisera raised against vertebrate GH. A peptide cross reacting with human, bovine and cGH monoclonal antibodies has also been demonstrated in insect (*Locusta migratoria*) brain (Vanden Broeck et al, 1990) and in molluscs (Moriyama et al, 1990).

Endocrine glands are not developed in some invertebrates, so these peptides must be produced in other organs or tissues. It has therefore been proposed that peptides and other elements such as receptors, arose in microbes and were carried in darwinian fashion to multicellular organisms in the process of evolution. Thus, intercellular communication phylogenetically arose from local tissue factors acting on the secretory cells themselves and on neighbouring cells. Later these molecules developed into hormones and neurotransmitters concomitant with the evolution of morphological and developmental endocrine structures of the organism. This theory also provides a rationalization for the multiple modes of action possesed by chemical messengers, which may act as hormones, neurotransmitters, autocrine/paracrine agents and local tissue factors (Roth et al, 1982). Thus, in a multicellular organism it is the anatomical relationship between the secretory cell and its target cells that has undergone extensive evolution (Krieger, 1983; Roth et al, 1983; Brinton et al, 1987), whereas the chemical messengers and cellular response induced by transmitter occupying its receptor are more ancient and highly conserved.

It also suggests that the synthesis and secretion of GH by the pituitary gland should be considered the unusual situation rather than the rule. Further evidence for

immune cells as an ancestral site for GH production is provided by the studies of Ottaviani et al (1992). Hemocytes of the primitive invertebrate *Viviparus ater* were shown to contain ir-GH like molecules. These cells are the ancestral cells responsible for defence mechanisms in these gastropods, and have phagocytic activity and surface receptors for con-A (Ottaviani et al, 1989). These cells were also proposed to belong to the APUD series described by Pearse (1984). Recent studies have shown that many polypeptides of the APUD cells are shared by neural and endocrine systems, and this may be due to a common origin of these cells from the neuroectoderm (Pearse, 1984). Production of GH-ir by this ancestral immune cell suggests that the immune system is also involved in the diffuse neuroendocrine system. This may also be true for vertebrates as a large part of the thymus gland (at least in birds) may contain neurally derived tissue (Hammond, 1954; LeDouarin, 1974; Bulloch, 1987). Epithelial cells in the thymus are chromagranin positive and specific staining for this protein has also been demonstrated in spleen cells (Hogue Angeletti and Hickey, 1989). This substance is a highly acidic calcium binding protein which is a neuroendocrine cell marker. The association of a neuroendocrine-type cell with immune tissues may therefore provide a mechanism linking nervous and immune systems. Furthermore, some macrophage cells may originate from brain parenchyma (Giulian, 1987), where they are involved in repair to brain injury (Berkenbosch, 1989). This may represent a further integrated pathway for interaction between nervous, endocrine and immune systems and indicates the overlap between these systems.

4.3. Ectopic GH Gene Expression

The regulation of GH gene expression in these ectopic tissues should be considered. The pituitary specific transcription factor Pit-1/GHF-1 was thought to be responsible for restriction of GH gene expression to somatotrophs of the anterior pituitary gland (Theill & Karin, 1993). Although this factor is present in immune cells (Delhase et al, 1993), its absence from the brain suggests different regulatory influences are at work in ectopic sites (Emanuele et al, 1992; Delhase et al, 1993). The cGH promoter may not be regulated by a Pit-1/GHF-1 transcription factor as its structure did not share homology with rat or hGH promoter sequences (Tanaka et al,

1992). The lack of a pituitary specific transcription factor may therefore be responsible for the ectopic transcription of the GH gene in the chicken. However, relatively little is known about the physiology and biochemistry of regulatory factors involved in expression of the GH gene in the chicken. As GH mRNA could not be detected in extra-pituitary tissues using Northern hybridization, this precludes investigations of regulatory influences on expression of the GH gene in these ectopic sites. Other techniques are available to quantitatively examine gene expression, including quantitative or competitive PCR and ribonuclease protection assays. These techniques, however, require lengthy preparatory steps for establishment and are difficult to characterize with regards to assay precision and accuracy. Quantitative PCR compares the amplification of an internal standard of known concentration, with the transcript of interest. As the PCR is an exponential process and small differences in efficiency between the primer pairs for the standard and the target mRNA could lead to large differences in yield of the PCR product, this technique is difficult to standardize.

4.4. Functional Implications

The presence of GH in the brain and lymphoid tissues suggests that GH has effects on neural and immune function. GH has central effects on brain size, DNA content, neurotransmitter levels and several aspects of behaviour (Sara et al, 1974; Noguchi et al, 1982; Kempf et al, 1985; Buntin and Figge, 1989; Astrom and Lindholm, 1990; Steger et al, 1991). The local synthesis of GH in the brain supports a paracrine or autocrine component in its mechanism of action. Furthermore, synthesis of GH in the avian hypothalamus where GH receptors have been demonstrated (Fraser et al, 1990) suggests brain GH may play a role in autoregulation of its own secretion via a short-loop negative feedback mechanism (Kracier et al, 1988). This is supported by the observation that neural GH may be regulated in an inverse fashion to pituitary GH (Hojvat et al, 1986).

As in the endocrine system GH actions in the immune system may be mediated by IGF-1 which has also been implicated as a haemopoeitic growth factor (Kelley, 1991). However, the demonstration of GH receptor gene expression in spleen and

thymus in the present study is suggestive of direct actions on immune tissues. The presence of GH-ir in these same tissues also indicates that GH may be acting via paracrine or autocrine mechanisms to influence immune function. These findings are in agreement with other studies in mammals which have demonstrated GH synthesis and secretion in cells of the immune system. This hormone-receptor system may be involved in proliferation of thymocytes (Berczi et al, 1991; Goya et al, 1992), T-lymphocytes (Geffner et al, 1990) and B-lymphocytes (Kimata and Yoshida, 1994). GH may also act directly on macrophages to stimulate superoxide anion synthesis and NK cells to stimulate production of IFN (Kiess et al, 1986). It is well established that GH plays a crucial role in thymic proliferation and hormone production. GH levels are strongly associated with thymic size and thymulin production in dwarf mice and pituitary deficient dogs (Pierpaoli et al, 1969; Roth et al, 1984), ageing dogs (Goff et al, 1987), GH-deficient children (Mocchegiani et al, 1990) and acromegalics (Mocchegiani et al, 1992; Timsit et al, 1992). Indeed, GH treatment stimulated thymulin production in aged dogs (Goff et al, 1987) and GH_3 cell implants reversed the thymic involution and decline in IL-2 production observed in aged rats (Kelley et al, 1986). Growth hormone may also be necessary for normal growth and maintenance of the bursa of Fabricius and secondary lymphoid organs such as the spleen. Growth hormone administration to chickens enhanced spleen weight (Haddad and Marshaly, 1991) and restored bursal weight in hypophysectomized and ageing chickens (Scanes et al, 1986), in addition to increasing the numbers of certain T-lymphocyte subsets (Marsh et al, 1992).

4.5. Conclusion

In conclusion, the synthesis of GH in extra-pituitary tissues indicates it may play many other, possibly ancestral roles in the chicken, and not simply be involved in the growth process for which it was named. The expression of GH and its receptor in lymphoid tissues suggests that GH exerts its effects in a paracrine or autocrine fashion in the immune system.

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